

IMPROVED PRODUCTION AND SECRETION OF CANCER TESTIS ANTIGEN
SSX2 IN DIFFERENT EXPRESSION HOSTS

A Dissertation

Presented to the Faculty of the Graduate School
of Cornell University

In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by

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August 2009

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IMPROVED PRODUCTION AND SECRETION OF CANCER TESTIS ANTIGEN SSX2 IN DIFFERENT EXPRESSION HOSTS

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Cornell University 2009

There is a great demand to develop new therapeutic strategies to treat cancers. Of the potential therapeutic strategies, cancer testis antigen SSX2 has shown promising effects for the treatment of different cancers. To produce the quantities of SSX2 required for the human Phase I/II proof-of-principle clinical trials, improved production and secretion of SSX2 were studied in two microbial systems, *Escherichia coli* and *Pichia pastoris*.

The first part of this work shows the development of a production process for SSX2 antigen from *E. coli* strain C41(DE3), resulting in a yield of 1.1 g L⁻¹ as inclusion bodies. Urea-solubilized SSX2 inclusion bodies were purified by immobilized nickel affinity, anion exchange and hydrophobic interaction chromatography. Purified SSX2 was characterized by ELISA and recognized by human sera containing anti-SSX2 antibodies.

In the second part of this study, further process development and production scale up of SSX2 were carried out in the Cornell/LICR cGMP facility. The yield, endotoxin content and purity of SSX2 were improved in the newly developed process. The result of a 10 L scale development run is also presented.

The third part of this work focused on the improvement of SSX2 secretion by *P. pastoris* as a means to produce a delayed-type hypersensitivity (DTH) skin test reagent for SSX2 cancer vaccine. SSX2 was minimally secreted by *P. pastoris*. Deletion of the SSX2 C-terminal nuclear localization signal (NLS) did however

improve secretion. NLS was responsible for accumulation and misfolding of SSX2 in endoplasmic reticulum (ER) and deletion of NLS improved SSX2 folding inside the ER to facilitate its secretion. Production of SSX2 without NLS was scaled-up, resulting in a yield of 21.6 mg L⁻¹.

The last part of this work reveals the proteome of different fermentation culture media of *P. pastoris* as a means to enhance recombinant protein purification and secretion. The study of the proteome of different *P. pastoris* cultures indicates that most proteins exhibit acidic isoelectric points including naturally secreted and intracellular proteins. This study provides host cell protein profiles in different fermentation culture media and suggests potential biotechnology applications based on the discovery of these proteins.

BIOGRAPHICAL SKETCH

The author, Chung-Jr Huang, was born to Yang-Ming Huang and Shu-Chen Tien, in Nantou County, Taiwan, on July 30, 1975. He attended the National Taiwan University and received a Bachelor degree in Forestry in 1997. During his time as an undergraduate student, he found his interest in Applied Microbiology. After graduated, he joined Food Microbiology lab at the same university under the guidance of Professor Cheng-Chen Chou and received a M.S. degree in 1999.

The same year after he graduated, he joined the Taiwan Army and served as a second lieutenant for a year. He then worked as a researcher at ChungHwa Chemical Synthesis and Biotech Corp., from year 2000 to 2004. There, he studied to improve and scale up production of different secondary metabolites used as medicines from different fungi. Upon awarding a scholarship from Ministry of Education, Taiwan, he, his wife, Man-Li Chang, and his daughter, Mei-Ching Huang, moved to Ithaca, NY, where he started his Ph.D. program in the department of Microbiology, having opted for Biomedical Engineering and Biochemistry, Molecular & Cell Biology as minors.

Under the guidance of professor Carl Batt, he has worked on improved production and secretion of cancer testis antigen SSX2 for human Phase I/II clinical trials. He also had an opportunity to be involved in Cornell University/Ludwig Institute for Cancer Research Partnership to work inside a cGMP facility to produce cancer vaccines in helping cancer patients.

To Man-Li

ACKNOWLEDGMENTS

I would first to acknowledge my advisor and mentor professor Carl Batt for his guidance and giving me the opportunity and freedom in my scientific pursuits. He had shown me the importance of my research and work, a critical driving force to keep me moving forward. I would also like to thank my committee members, Professor William Brown from department of Molecular Biology and Genetics and Professor Matthew DeLisa from the Department of Biomedical Engineering for their insights and support.

I thank Ministry of Education, Taiwan and the Ludwig Institute for Cancer Research for making this work possible. I also acknowledge Department of Microbiology, at Cornell University, for all the support. I thank Dr. Lloyd Old, Dr. Yao-Tseng Chen and Dr. Gerd Ritter for discovery, support and advice for studies related to CT antigens including SSX2.

Special thanks also go to colleagues involved in Cornell/Ludwig Institute for Cancer Research partnership, including Dr. Rishard Chen, Kyle Anderson, Brian Newton, Dr. Leonardo Damasceno, Denise Payne and Adam Lowe. A very special thanks goes to Leonardo Teixeira, for his support and insightful discussions.

I am also thankful to all Taiwanese friends I have met at Cornell University, especially to Yun-Wei, Chia-Chen, Ron-Bin, Tuo-Hung, Ming-Li and Guan-Hua. You have made my stay at Cornell so special.

Above all, I must acknowledge all the love and support I gained from my wife, Man-Li, who always there for me through thick and thin. A huge appreciation to my parents and parents in law for supporting me through the difficult time I had. To my daughter, Mei-Ching, thank you for giving mommy and me all the joys and happiness. Having you is the greatest thing in our lives.

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CHAPTER 1

INTRODUCTION

Recombinant protein expression in *Escherichia coli* and *Pichia pastoris*

***E. coli* as a protein expression host**

Recombinant DNA technology has changed the way to produce proteins and has increased their availability in research and industry. The very first biopharmaceuticals such as recombinant insulin and recombinant human growth hormone were produced from *Escherichia coli* and marketed in the early 1980s. Before this time, these proteins were only made available by extraction from natural sources. Since then, due to its well-characterized genome, mature tools for genetic manipulations and efficient expression of the target gene, *E. coli* has become an attractive expression system to produce heterologous proteins from different origins.

Several factors, such as strains, expression vectors, promoter strength, translation regulation, codon usage and fermentation strategies, have been reported to affect the level of recombinant protein expression in *E. coli* (40). Among strains, *E. coli* K12 and B strains, e.g. BL21, are most widely used to express non-glycosylated peptides and proteins. In recent years, pET vectors containing the T7 bacteriophage promoter (commercialized by Novagen, Madison, WI) gained increasing popularity for production of different recombinant proteins. Typically, this system requires a host strain lysogenized with a DE3 phage fragment, encoding the T7 RNA polymerase under the control of the IPTG-inducible promoter *lacUV5* (59). Because the T7 promoter is vigorously induced by, and very specific to, its own T7 RNA polymerase, high expression level of recombinant protein can be achieved and well-controlled. *E. coli* BL21(DE3) or its derivative C41(DE3) are usually used as the host in

combination with the pET vector system. Particularly, the C41(DE3) strain was found to be a better host for expressing toxic proteins compared to BL21(DE3) because of one uncharacterized mutation, preventing the cell death associated with the expression of toxic proteins. Later the mutations in the UV5 promoter governing expression of T7 RNA polymerase were found to be the key to the improved toxic protein overexpression in C41 strain (68).

The so-called codon bias also poses another challenge for expressing heterologous protein in *E. coli*. The frequencies with which genetic codons are used to translate protein vary significantly between different organisms. Preferred codons correlate with abundance of cognate tRNAs available within cells. Sometimes, the cells will also adjust protein expression level by using different genetic codons (36). The effect of codon bias on heterologous protein expression is obvious by decreasing the protein translation rate due to the rare codons used by *E. coli*. Fortunately, this problem can be addressed by changing the problematic codons to *E. coli* preferred codons, or by the coexpression of cognate tRNA genes such as coexpression with pRARE plasmid in expression strain (17). High cell density fermentation is normally used to increase the productivity of recombinant protein in *E. coli* using a fed-batch feeding strategy. Different feeding controls have been developed including DO-state and pH-state controls to avoid overfeeding that accumulates acetic acid in the culture media, inhibiting both cell growth and recombinant protein production (44).

Expression of recombinant protein can be either in the cytoplasm or be secreted to periplasm and extracellular space. In cytoplasmic expression, protein can be soluble or aggregated as inclusion bodies (IB) (Figure 1-1), which depends on intrinsic property of protein sequence or other cultivation parameters such as promoter strength and cultivation temperature (43). IB formation is mainly due to inappropriate

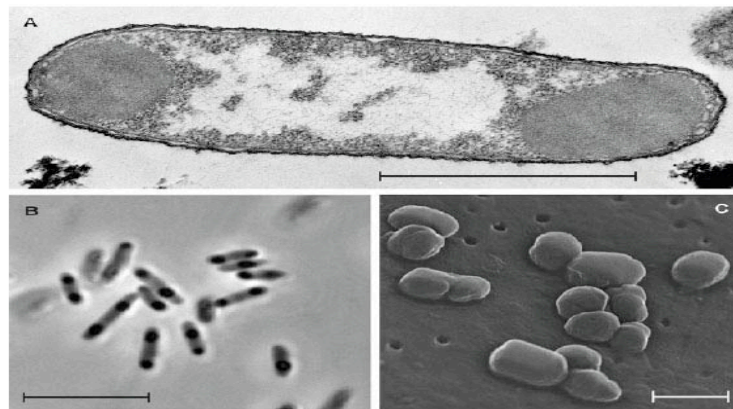


Figure 1-1. Images of *E. coli* inclusion bodies. Visualization of inclusion bodies in *E. coli* producing elongation factor Tu (EF-Tu) of *Streptomyces aureofaciens* by transmission electron microscope (A; bar = 1μm), phase-contrast optical microscope (B; bar = 5μm) and scanning electron microscope (C; bar = 1μm) of purified inclusion bodies. (Nguyen et al., 2005)

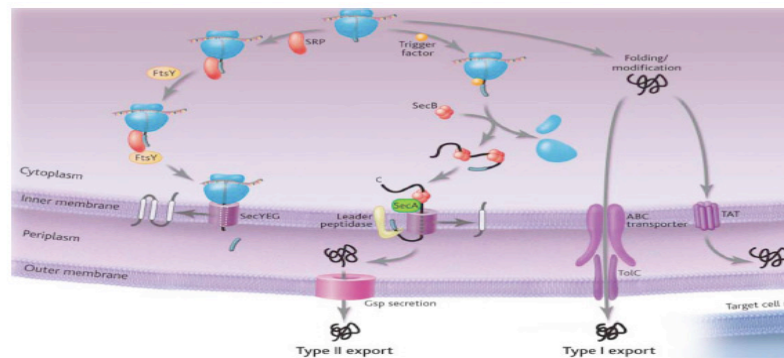


Figure 1-2. Recombinant protein secretion pathways in *E. coli*. Three different secretion pathways, type I, type II and TAT, which have been used for recombinant protein secretion in *E. coli* either to extracellular space or periplasm. (Wickner and Schekman, 2005)

folding and overexpression of recombinant protein. Most IB-forming proteins require solubilization in chaotropic agents such as urea and guanidinium chloride, then refold. However, some vaccines like NY-ESO-1 are formulated in 4M urea which do not require protein refolding upon immunizing cancer patients (4, 51). Gram-negative bacteria have generally used five different secretion pathways for secreted proteins such as degradative enzymes, toxins and pathogenic factors. Among them, type I mechanisms have been reported to secrete different recombinant proteins in *E. coli* to extracellular space. Type II secretion have been successfully explored to transport recombinant proteins to periplasm. However, it is rarely used for extracellular secretion as it has a very narrow substrate specificity for translocation across the outer membrane (49). More recently, a novel twin-arginine translocation (TAT) system was discovered and used for secretion of several recombinant proteins including antibody fragment (Figure 1-2) (24). One of the advantages for secreting recombinant protein in *E. coli* is that protein is generally correctly folded into its native state where protein refolding is not necessary. However, the yield is usually low compared to IB and improved secretion requires overexpression of some periplasmic factors or chaperones and use of the leaky strains (49).

Even though *E. coli* has been considered as an excellent recombinant protein expression host, there are some disadvantages in this system such as inability to perform posttranslational modifications, inefficient secretion machinery and presence of endotoxin in the final purified recombinant protein. Endotoxin is the lipopolysaccharide (LPS) of gram-negative bacteria and its high concentration in the recombinant therapeutics can cause a variety of inflammatory responses in humans (28). Therefore, endotoxin level is considered an important quality criterion for *E. coli*-derived recombinant therapeutics. The limit of endotoxins is set at 5 endotoxin

units (EU) per kg patient weight and hour for both intravenous and intramuscular applications according to United States Pharmacopeia. One EU is roughly equivalent to 100 pg of endotoxin. Therefore, the amount of the endotoxin that can be administered to a patient is depended on the therapeutic dose and the endotoxin concentration of the therapeutics. The disadvantages for using *E. coli* as a expression host have been constantly challenged and addressed by scientists. For example, the first discovered N-linked glycosylation pathway in bacteria *Campylobacter jejuni* has been engineered into *E. coli* to produce recombinant glycoprotein (67). Recent advances in understanding protein secretion in bacteria also show great promise leading to new strategies to facilitate the secretion of recombinant protein (31). Several technologies such as anion exchange chromatography, positive charged depth filters, and detoxification of endotoxin by modification of its structure by overexpression of *H. pylori* genes have been developed and studied to remove or detoxify endotoxin from recombinant protein solutions (14, 47).

***P. pastoris* as a protein expression host**

The methylotrophic yeast *Pichia pastoris* was also widely used for high yield expression of various recombinant proteins by secretion into the culture medium or intracellular localization. *P. pastoris* has evolved a specific pathway with unique enzymes such as alcohol oxidase, catalase and dihydroxyacetone synthase to utilize methanol as the sole carbon and energy source. The reactions occur in peroxisomes followed by metabolic steps in cytoplasm to dissimilate methanol (Figure 1-3) (13). There are two genes that encode alcohol oxidase: *AOX1* and *AOX2*. *AOX1* accounts for the majority of alcohol oxidase activity in cells, as it has a very strong and tightly regulated promoter. Transcription of *AOX1* is repressed with the presence of other carbon sources such as glucose and glycerol. When cells are grown in the medium

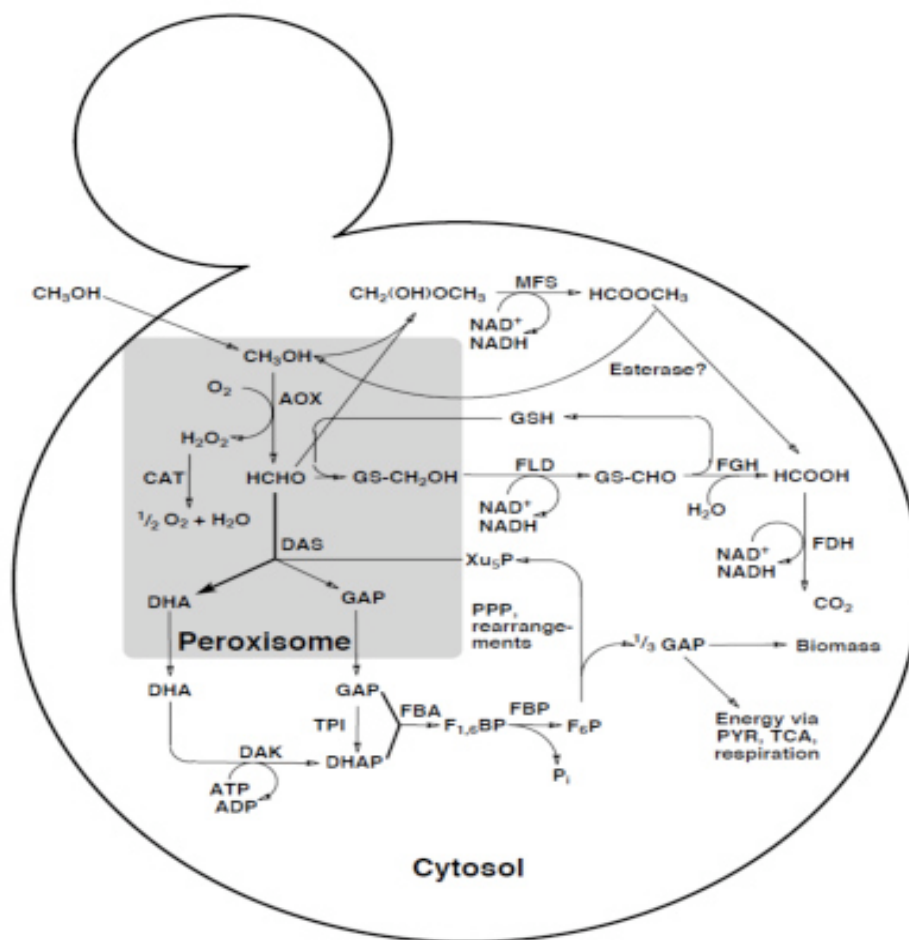


Figure 1-3. Methanol utilization pathway in methylotrophic yeasts including *P. pastoris*. The key enzymes involved in methanol metabolism are: AOX: alcohol oxidase, CAT: catalase, DAS: dihydroxyacetone synthase, and FDH: formate dehydrogenase. (Hartner and Glieder, 2006)

where methanol is the only carbon source, expression of *AOX1* is induced where alcohol oxidase can comprise over 30% of the total intracellular proteins and peroxisomes can account for up to 80% of cytoplasmic space (32).

Over 400 recombinant proteins have been produced in *P. pastoris*. Since *P. pastoris* is also a eukaryote, it provides the potential to produce soluble and correctly folded recombinant proteins and offers significant advantages over the *E. coli* expression system for the production of many heterologous eukaryotic proteins (11). A number of different *P. pastoris* strains with various genotypes are available. X-33 is the wild type strain where both *AOX1* and *AOX2* are functional to utilize methanol and is designated as Mut⁺ strain, i.e. methanol utilization plus strain. The KM71H strain, however, contains a non-functional *AOX1* and relies on the *AOX2* gene to produce alcohol oxidase, leading to a slow growth on methanol-containing media. Its phenotype is termed methanol utilization slow (Mut^s). The SMD1168H strain is defective in the vacuole peptidase A (*pep4*) which is responsible for activation of carboxypeptidase Y and protease B1. This strain is used to reduce the proteolytic activity for some recombinant proteins. Strains such as GS115, SMD1168 and KM71 are defective in the histidine dehydrogenase gene (*his4*) and can be selected on their ability to grow in non-histidine containing media (21). Depending on the goals and types of recombinant proteins, these strains provide useful tools to improve recombinant protein expression.

The *AOX1* promoter has been the most widely reported and used of all the available promoters in *P. pastoris* (12). Because alcohol oxidase has a poor affinity for oxygen, *P. pastoris* can compensate its ability by an immensely upregulated *AOX1* promoter when methanol is the only carbon source. This promoter can drive high levels of recombinant protein production with a single integrated copy of the

expression cassette. Another advantage of this promoter is that it can be switched on and off by manipulating the presence of different carbon sources in the culture media. The constitutive promoter, *GAP* (glyceraldehyde 3-phosphate) promoter, is also available in *P. pastoris* without the need of methanol induction to produce recombinant proteins. Different carbon sources, such as glucose, glycerol, oleic acid and methanol, can be used to culture the cells to express proteins. Several studies have shown some proteins achieved high levels expression by using the *GAP* promoter (70). However, one general advantage of using the *GAP* promoter instead of the *AOX1* promoter is that methanol, which is considered a fire hazard and contaminant for the production of recombinant proteins, is not required.

Both intracellular expression and extracellular secretion of recombinant protein are applicable in *P. pastoris*. This usually depends on the protein to be expressed. If the target protein does not secrete in its native form, forcing its secretion through the folding pathway may result in misfolding or alter its properties due to incorrect post-translational modifications. Indeed, many proteins have been expressed successfully in the intracellular milieu in *P. pastoris*, particularly for some membrane-associated proteins such as the hepatitis B surface antigen (66). However, it is difficult to purify the target protein as it typically represents less than 1% of the total intracellular proteins (21). On the other hand, the secretion of recombinant protein into the culture media offers several advantages such as avoiding toxicity of intracellular accumulated recombinant protein and simplification of the protein purification. In addition, entering of the protein through secretory pathway allows post-translational events such as glycosylation, proteolytic maturation and disulfide bond formation. These properties are necessary for some recombinant mammalian proteins to ensure their biological activity (46). In fact, the secretion of properly folded recombinant proteins is one of

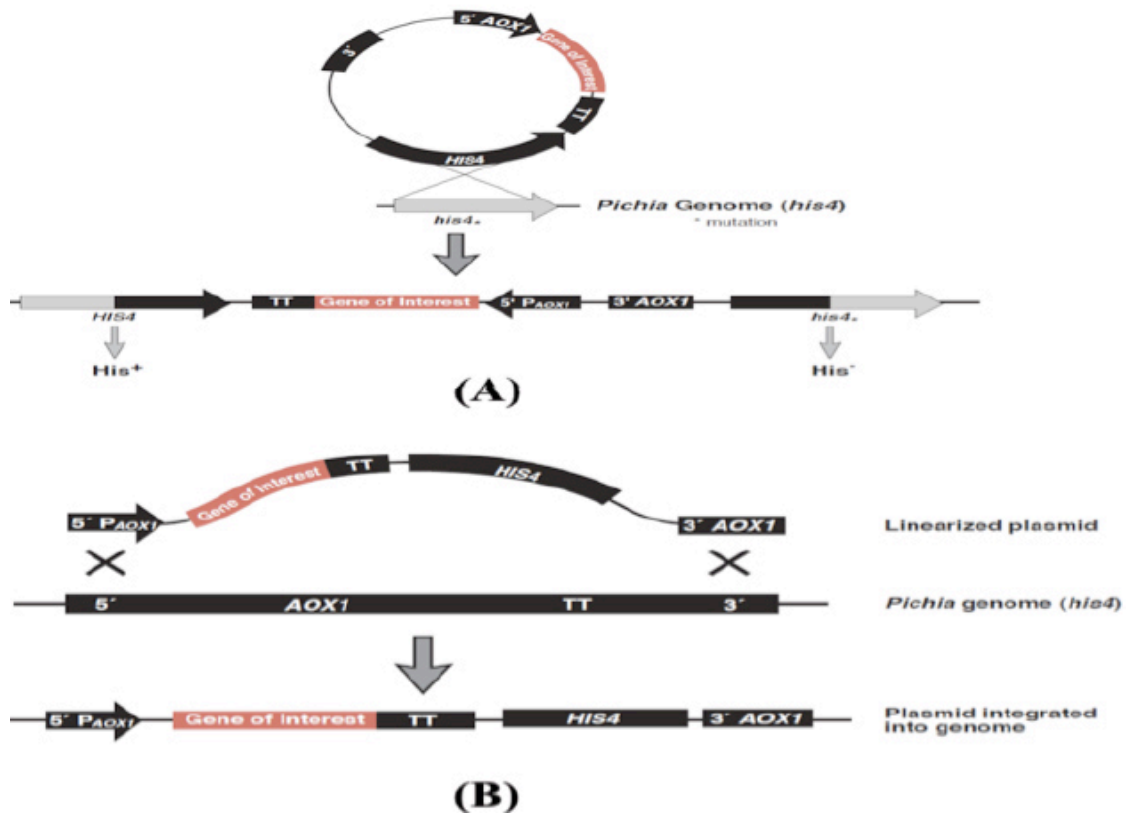


Figure 1-4. Examples of homologous recombination of expression cassettes into *P. pastoris* genome. (A) Gene insertion occurs at the *his4* locus of *Pichia* genome by single cross over event between *HIS4* gene on the expression plasmid, resulting in a *His4⁺* transformant. (B) Gene replacement is achieved by double cross over event between the upstream and downstream *AOX1* gene of the genome and the expression vector. This results in the complete elimination of *AOX1* gene coding region, resulting in a *His4⁺* and *Mut^s* transformant. (*Pichia* Manual, Invitrogen)

the crucial factors taken into account when yeast is considered as a preferred expression host. Different secretion signals, including alpha-mating pre-pro leader sequence (α -MF), acid phosphatase signal (PHO) and invertase signal (SUC2), have been used to secrete recombinant protein in *P. pastoris*. However, there is no general rule of which leader signal is the best signal for recombinant protein secretion. Instead, trial and error experiments are often required to identify the optimal secretion signal for a specific recombinant protein.

Once the expression vector is constructed, it is integrated into *P. pastoris* genome at the specific locus by homologous recombination to generate genetically stable transformants (Figure 1-4). Integration can occur via gene insertion or gene replacement in either a marker gene (e.g. *HIS4*, *ARG4* and *URA3*) or the *AOXI* promoter. Integration by gene insertion occurs from a single crossover event at a high frequency (50-80% of His⁺ transformants) and tandem multiple recombination events can be achieved at the rate of 1-10% of transformants to generate a multicopy strain (19). To obtain a gene replacement transformant, an expression vector requires digestion and linearization to have double crossover events. This occurs at a frequency of 5-25% of total transformants. The remainder of the transformants are the result of gene insertion (53). Since the expression cassette is integrated into the *Pichia* genome, there is no need to add antibiotic to maintain the transformed population cells. Transformed cells can be selected by either auxotrophic complementation, or resistance to antibiotics (e.g. Kanamycin and Zeocin). The latter is used to collect multicopy transformants, where the levels of drug resistance correlate to the expression cassette copy number.

To improve recombinant protein production in *P. pastoris*, fermentation is essential as cell density usually correlates with high protein yield. *Pichia*

fermentations are characterized by high cell densities, short fermentation times and the use of chemically defined media. It can be categorized into two types of fermentation, one-phase and two-phase, which depends on the types of promoter used. When the constitutive *GAP* promoter is used, one carbon source (glucose or glycerol) is used by the cells during the fermentation. While using the *AOXI* promoter, cells are grown in glycerol until it is depleted, then switch to the second phase where gene expression begins by feeding methanol into the fermentor. There are several factors affecting protein production yield including composition of culture medium, strain, pH, dissolved oxygen level, temperature and feeding strategy where the best fermentation conditions usually require optimization of these parameters (45). For example, several studies have demonstrated that some recombinant proteins, such as insulin-like growth factor and A33 single chain antibody (A33scFv), are more stable and produced in high yield at pH 3.0, rather than at a higher pH (22).

To maintain methanol concentration within optimal limits, three different methanol-feeding strategies are generally used to achieve high cell density without hampering cell growth. First, the methanol-feeding rate is controlled according to the methanol concentration in the culture media (22). Second, the methanol-feeding rate is adjusted by the dissolved oxygen level by increasing feeding rate when dissolved oxygen concentration increases, and *vice versa* (50). The final strategy is to control the feeding rate according to the specific growth rate during the induction phase (20). Successes of different strategies have been reported from different fermentation cultures expressing different recombinant proteins (45).

Although the *P. pastoris* expression system has the benefits of eukaryotic folding pathways, its glycosylation machinery is different than humans and other mammalian systems. This characteristic impedes recombinant glycoprotein production

in *P. pastoris* because correct glycosylation pattern is important to maintain biological activity, stability and low immunogenicity of some proteins (21). Like other mammalian cells, yeast N-glycosylation is initiated in the endoplasmic reticulum (ER) and then the substrate is transported into the Golgi apparatus for additional trimming of the glycan. It is at the Golgi apparatus that yeast display different glycosylation pathways, resulting in the production of mostly high-mannose containing oligosaccharides. Due to the lack of α -1,3-mannosyltransferase in the Golgi complex, *P. pastoris* has much shorter mannose-containing glycans than those expressed in *S. cerevisiae* (71). However, mannose-containing glycan is a non-human glycosylation and hence immunogenic, which might cause adverse immune responses. Recently, scientists have showed that it is possible to engineer the glycosylation pathway in *P. pastoris* to produce humanized glycoprotein by replacing endogenous glycosylation enzymes with five eukaryotic enzymes to produce complex human N-glycan protein (8). This technology further expands the application of using *P. pastoris* for recombinant protein expression.

Cancer testis antigens

Identification of cancer testis (CT) antigens

In the early 1990s, Boon and his colleagues reported isolation of a human tumor, termed melanoma antigen-1 or MAGE-1, from a melanoma cancer patient who elicited cytotoxic T lymphocyte (CTL) responses that recognized autologous tumor cells (64). The expression of MAGE-1 was later found not only in melanoma but also in other cancer types. In normal tissues, its expression was ordinarily restricted to the testes (25). The technique used in studying this antigen was called T-cell epitope

cloning and led to the identification of similar tumor antigens such as B melanoma antigen (BAGE) and G antigen (GAGE) (65). Although identification of these new antigens provides potential targets for immunogenic cancer vaccines, this approach was technically challenging.

An approach developed by Sahin and colleagues called SEREX (serological analysis of recombinant tumor cDNA expression libraries with autologous serum) circumvents the limitations of T-cell epitope cloning in identifying more tumor antigens (Figure 1-5). It was designed to isolate tumor antigens that elicit high titer immunoglobulin G (IgG) responses in cancer patients (55). The first gene identified by SEREX was HOM-MEL-40 or synovial sarcoma X breakpoint 2 (SSX2) from a melanoma patient (54). Analysis of SEREX protocol on esophageal cancer led to the discovery of NY-ESO-1 antigen, the most immunogenic tumor antigen known to date identified by SEREX (15). Like MAGE-1 and BAGE, SSX2 and NY-ESO-1 were also restricted in their expression to testes and different cancers. These characteristics were shared by unrelated genes among other tumor antigens. Therefore, Chen and his colleagues proposed the term cancer testis (CT) antigens to this type of tumor antigen (55). The immunity generated from the CT antigens in cancer patients can be cellular or humoral responses. Some antigens elicit both cellular and humoral responses.

Based on the definition of CT antigens, several techniques including representational difference analysis (RDA), differential display, cDNA oligonucleotide array analysis and bioinformatic analysis, have been applied to identify CT antigens (55). To date, according to the published literature, there are over 130 genes identified as CT antigens that belong to 83 gene families with some of their functions unknown (38). By comparing CT antigen expression data from different studies, most of them shared these characteristics including: 1) expression restricted to

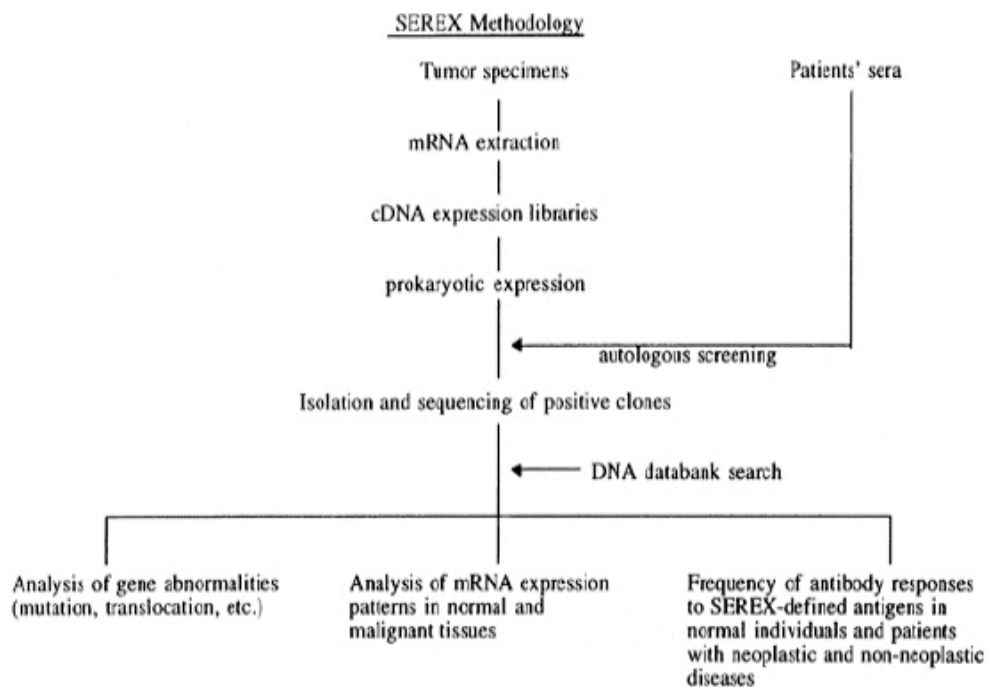


Figure 1-5. Illustration of SEREX protocol for cloning and identifying human cancer antigens eliciting high immunoglobulin G (IgG) antibodies. (Chen, 2004)

gametogenic tissues and cancer; 2) coding genes frequently map to chromosome X; 3) exist as multigene families; 4) immunogenic in cancer patients; 5) heterogeneous protein expression in cancer; 6) expression may be associated with tumor progression and with tumors of high metastatic potential; and, 7) *in vitro* activity by hypermethylation and/or histone deacetylase inhibitors (55). Importantly, CT antigens are immunogenic in cancer patients with restricted expression, and as the result, have the potential to be used as a cancer vaccine.

The mechanism of cancer immunotherapy by CT antigens

CT antigens represent the ideal targets for cancer immunotherapy, a process that the immune system can be trained to attack tumors in the same way that it targets the infectious agents. This relies on administration of CT antigens to cancer patients to improve their immunity against cancer cells to kill cancer cells. In general, antigen or peptides will be delivered to antigen presenting cells such as Dendritic Cells (DC) that will degrade the antigen and display as epitopes on the cell surface through a pathway called Major Histocompatibility Complex (MHC) class I pathway. DC will later present the epitopes to T cells to activate and proliferate tumor-specific cytotoxic T lymphocytes (CTL) which can target and kill the cancer cells (Figure 1-6) (29). Since testis germline cells are MHC class I negative, the expression and activation of CT antigen-specific T lymphocytes is highly tumor specific, making CT antigens ideal targets for cancer immunotherapy.

In the initial clinical trial with MAGE-A3 peptides in HLA-A1 patients, 7 out of 25 patients showed significant tumor regression (48). The results of the Phase II proof-of-concept clinical trial of MAGE-A3 in MAGE-A3 positive patients with stage IB or II Non-Small Cell Lung Cancer was promising, and moved on to the Phase III trial in 2008 (10). Induction of CD4⁺ and CD8⁺ T cells specific to a broad range of NY

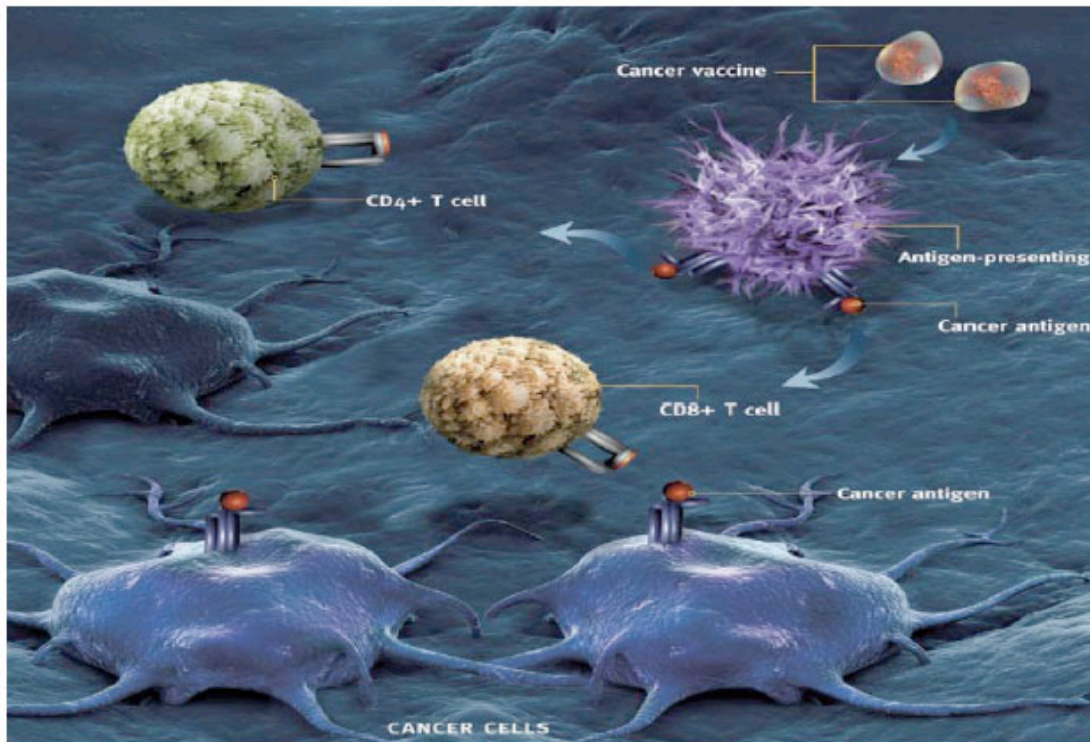


Figure 1-6. A simplified cartoon illustration of cancer immunotherapy by CT antigens (cancer vaccines). The vaccines will be delivered to antigen-presenting cells such as dendritic cells to activate the antigen-specific T lymphocytes to kill cancer cells.

-ESO-1 epitopes were found in the data from its several ongoing clinical trials against different cancers (27). These examples show great promise for the use of CT antigens as cancer vaccines, and their successes seem to rely on the identification of appropriate antigens and the effective immunization protocol.

SSX2 as a cancer testis antigen

SSX2 is a CT antigen that is expressed in a wide variety of tumors including human melanoma, colon cancer, hepatocarcinoma, breast carcinoma (60, 61). Its gene is located on X chromosome with at least four other similar genes (*SSX1*, *SSX3*, *SSX4* and *SSX5*) (35). SSX2 has been shown to elicit both spontaneous humoral and cellular immune responses in cancer patients. By analyzing a tumor-infiltrated lymph node of a melanoma patient bearing an SSX2-expressing tumor, Ayyoub and colleagues have recently identified the first SSX2-derived CD8⁺ T cell epitope, that corresponds to the peptide SSX2₄₁₋₄₉, and is recognized by specific CTL in an HLA-A2-restricted fashion. This study also suggested that SSX2₄₁₋₄₉-specific CTLs exhibit high tumor reactivity, and are selectively expanded during immune responses to SSX-2-expressing tumors *in vivo* (3). Other SSX2-derived T cell epitopes recognized by CD4⁺ T cells have also been reported (2). SSX2-specific CD8⁺ T cells isolated from hepatocellular carcinoma patients can specifically and efficiently kill tumor cells *in vitro* (9). These studies have demonstrated that SSX2, like MEAG-A3 and NY-ESO-1, is a promising cancer vaccine for cancer immunotherapy.

Other than being a CT antigen, SSX2 is also involved in a chromosomal translocation t(X;18)(p11.2;q11.2) characteristically found in 70% of human synovial sarcoma (18). The translocation results in a synovial sarcoma translocation gene (*SYT*)

fused most C-terminal SSX2 to the C-terminus of SYT to form SYT-SSX2 fusion believed to be a possible transcription activator for aberrant transformation of synovial sarcoma (52). SSX2 is a nuclear protein containing a C-terminal bipartite nuclear localization signal (NLS) that is responsible for the translocation of SYT-SSX2 and SSX2 to the nucleus (26). Inspections of the SSX2 sequence revealed a high content in charged amino acid and an acidic C-terminal tail that is highly conserved among the SSX protein family and a predicted hydrophilic protein (Figure 1-7) (23).

Recombinant protein purification and process development

Protein purification

Expressed recombinant proteins from microbial or mammalian cells are required purification by downstream processing (DSP). This process is critical and challenging, especially for a therapeutic protein which usually requires over 99% purity with biological activity. DSP contains several purification steps including cell separation or lysis, preparative buffer exchange and several chromatographic steps. Among these processes, chromatography is the most efficient technique to separate the recombinant protein from the host proteins, however, its development has traditionally relied on trial and error approaches. Accordingly, large numbers of laborious runs and fraction analyses, from several hours to days, may be required to develop a successful purification methods for a specific protein.

The rule of thumb of recombinant protein purification includes four steps: clarification and concentration, primary contaminants removal, intermediate purification and polishing (Figure 1-8). For the first step, a centrifuge is usually used to collect the cells or supernatant, depending on localization of expressed protein. Cell

lysis is required if the protein is expressed in an intracellular manner. The use of membrane technology to remove contaminants or perform buffer exchange by cross flow filtration usually follows (63). The goal of primary contaminant removal is to provide a significant increase in the purity of the recombinant protein with respect to the starting material. There are several techniques applied in this step, including protein precipitation and affinity chromatography. For IB, high *g* force centrifugation and cross flow filtration are often used for primary contaminants removal. Intermediate purification is usually achieved by the combination of chromatography, cross flow filtration and other filtration steps (58). The chromatographic methods used in this step are ion exchange (IEX) and hydrophobic interaction (HIC) chromatography with the characteristics of high protein binding capacity and flow rate. Polishing steps are used to provide a final product with correct quality and purity prior to formulation. The targets for separation are usually isoforms, aggregates and other chemicals of the final products. Therefore, high resolution but low capacity size exclusion chromatography (SEC) is usually used for this step. Affinity and other chromatography might also be used to improve the purity or remove contaminants at the last polishing step (14).

Process Development

In the process of protein therapeutic discovery to administration in the clinic, development scientists are usually responsible for protein expression, fermentation and purification to provide clinical grade material for Phase I/II safety and proof-of-concept trials (5, 33, 39). Development scientists usually work on both the lab and manufacturing scale to optimize recombinant protein production processes to meet ambitious timelines and maintaining standards. Design of a productive and economical process requires not only a thorough understanding of the nature of the target protein,

but also establishing platform technologies. The latter includes rapid generation of an expressing system, various fermentation protocols and different generic purification processes for different proteins such as antibodies, inclusion bodies and secreted therapeutics (33). The key to the success of a process development effort is usually achieved when an interdisciplinary team of scientists, engineers and analytical experts working closely together.

Proteomic study and its applications in recombinant protein production

Proteomics: its technology and development

Proteomics is the study of protein properties (expression level, post-translational modification, interactions etc.) on a large scale to obtain a global and integrated view of disease processes, cellular processes and networks at the protein level (7). Proteomics is technically complicated because it includes the characterization and functional analysis of all proteins that are expressed by a genome. It is also very dynamic because protein expression levels depend on complex regulatory systems. The core technologies of proteomics include protein separation, sample preparation and identification. Two-dimensional polyacrylamide-gel electrophoresis (2D PAGE) has remained the most efficient and economic way of separating complex protein mixtures (7). Protein sample preparation can represent a challenge for current proteomics techniques, especially for proteins occurring at a low concentration. These proteins are difficult to recover in the processes of precipitation, digestion, gel extraction (16). Addition of a liquid chromatography (LC) step can complement this limitation to increase sample detection sensitivity. Mass spectrometry is used for protein identification, and the success in the identification may vary with

the sensitivity of the mass spectrometer, the completeness of the database, the presence of isobaric masses, post-translational modifications and other factors (30).

Two peptide ionization methods, matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), demonstrating the high sensitivity and ability of less chemical decomposition, have mainly been used for protein identification in proteomics (7). MALDI time of flight mass spectrometry (MALDI-TOF-MS) is an efficient and robust approach for high throughput protein identification. With the automation and the software available, over 1000 protein spots can be measured by one person per day (42). However, there are two major limitations for MALDO-TOF MS. First, it is not sensitive enough to identify low molecular weight proteins, which generate fewer peptides for identification. Second, it can not identify multiple proteins of a mixture. Protein samples or a separated 2D gel spot usually contain more than one protein, hence some proteins will be missed (30). A more comprehensive approach to identify proteins is to perform tandem mass spectrometry (MS/MS) which normally results in protein identification even with one peptide precursor ion. MS/MS also can confirm MS-based protein identifications. Another critical advantage for tandem mass spectra is that they allow the sequencing of amino acids for the unidentified peptides or peptides belonging to hypothetical proteins. In such cases, the amino acid sequences are subjected to a BLAST search for protein identification purposes (30).

Several protein identification techniques such as, LC-MALDI-TOF MS, LC-MALDI-TOF/TOF, LC-ESI-MS/MS (Figure 1-9), can be used based on different purposes. Once the peptide masses of the generated mass spectra have been determined, proteins related to these peptides can be identified by software such as MASCOT and SEQUEST by comparing masses of peptides generated from a specific

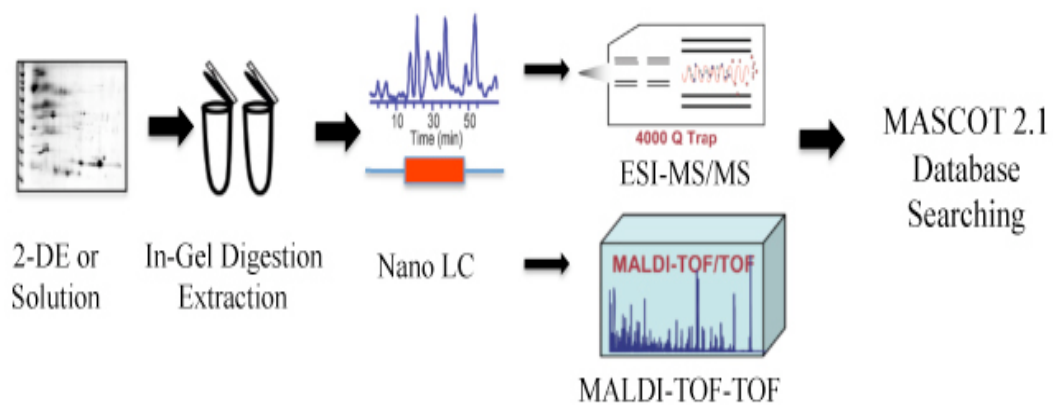


Figure 1-9. Summary of common steps in proteomic analysis for protein identifications from 2D gel spots or protein solution.

database. In some cases, many peptides fail to be identified mainly due to an incomplete database. The MS BLAST homology search can be used to overcome specific limitations imposed by mass spectrometric data and database availability (56).

Proteomics and process development

Proteomics techniques and studies have been applied in process development to improve recombinant protein production (34). Smales and colleagues have conducted a proteomic study of different IgG-producing GS-NS0 cell lines. In this study, a significant increase of host proteins, including immunoglobulin binding protein (BiP), endoplasmic reticulum chaperone and protein disulphide isomerase, was correlated with monoclonal antibody (MAb) productivities (57). Proteomic profiling of different batches of fetal bovine serum (FBS) have found additional growth-related proteins in FBS can improve cell viability (72). This information will aid in engineering high-titer strains and optimizing culture media to improve recombinant protein production.

Proteomic analysis of host cell proteins (HCPs) also plays a vital role in cell line selection and downstream processing (69). By comparing HCP patterns of different cell lines, scientists will be able to select a better protein expression strain in perspective of recombinant protein purification. Similar ideas can also be applied to select better cultivation conditions (41, 62). Identification of HCPs can reveal the identities of crucial contaminants with regard to the recombinant protein, providing strategies to remove these contaminants from the final product. Analysis of the proteome of secreted proteins (secretome) will also help to identify new secretion signals from secreted proteins. Although several studies have demonstrated that secreted proteins and their secretion signals can be predicted by analysis of the genome sequence, it usually lacks proteomic validation (1). However, the analysis of the proteome from the extracellular space normally contains some intracellular

proteins. For example, proteomic analysis of the extracellular proteome fraction of *Corynebacterium efficiens* YS-314 had identified 58 proteins. Among these proteins, 34 were secreted proteins with secretion signals predicted by SignalP, a software predicting the presence and location of signal peptide cleavage sites in amino acid sequences in different organisms (6). The rest of the proteins could be secreted protein by an unconventional secretion pathway or intracellular proteins (37).

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CHAPTER 2

EXPRESSION AND PURIFICATION OF THE CANCER ANTIGEN SSX2: A POTENTIAL CANCER VACCINE

Abstract

SSX2 is a cancer testis antigen expressed in a wide variety of cancers, including synovial sarcoma and melanoma. It holds promise as a potential antigen for cancer immunotherapy. A process for the production of recombinant SSX2 was developed by overexpressing a His-tagged fusion protein of SSX2 in *Escherichia coli* C41 (DE3). A T-7 promoter system was employed and a plasmid was introduced into the strain to compensate for rare codons in the SSX2 sequence. The production of SSX2 was scaled up to a 2 L fermentation that was operated under fed-batch conditions to improve productivity. After 32 hr cultivation, the wet cell mass reached 260 mg/mL, with SSX2 produced mainly as inclusion bodies at a concentration of 1.1 g/L. Urea-solubilized SSX2 was purified by nickel affinity, ion exchange and hydrophobic interaction chromatography. The recovery of SSX2 was 20%, and over 87% purity was obtained with an endotoxin level of 0.11 EU/μg. The purified recombinant SSX2 was characterized by ELISA and was shown to be recognized by human sera that have been reported to carry anti-SSX2 antibodies.

*Originally published as: Huang, C-J., Chen, R., Vannelli, T., Lee, F., Ritter, E., Ritter, G., Old, L. J., Batt, C. A. "Expression and purification of the cancer antigen SSX2: A potential cancer vaccine." 2007. Protein Expression and Purification 56:212-219. Reprint with permission from Elsevier.

Introduction

SSX2 was first identified by serological analysis of recombinant expressed clones (SEREX) from a cDNA library derived from a human melanoma (18). The SSX2 gene is located on chromosome 18 (Xp 11.22) fused to the 5' sequence of SYT, a gene encoding a putative transcriptional activation protein. Further studies found that the SSX2 gene was not only expressed in human melanoma but also in other malignancies, including colon cancers, hepatocarcinomas and breast carcinoma (18, 26). Expression studies in normal tissue showed that SSX2 transcripts were found mainly in testis tissue with low levels in the thyroid (8). In addition, different types of cancers elicit both humoral and cellular immune responses against the expressed SSX2 antigen (2, 4, 16, 18, 20). Since it is not expressed in normal tissue except in testis, but is in different malignancies and antibodies are found against it in some cancer patients, SSX2 is characterized as a cancer testis (CT) antigen (19).

CT antigens are tumor antigens exclusively expressed in tumor cells that also elicit immunogenicity in cancer patients. CT antigens are not expressed in normal tissues with the exception of testis germ cells and occasionally in female reproductive organs (19). Fortunately, testis germ cells are known to be MHC class I negative which are not recognized by T cells. Thus, CT antigens are highly tumor-specific and considered as an ideal cancer vaccine target for cancer immunotherapy (29). To date, several CT antigens, including NYESO-1, MAGE-3 and SSX2, have been identified and proven to elicit spontaneous humoral and cell mediated immune responses (19). Clinical trials of NYESO-1 and MAGE-3 as cancer vaccines have shown promising results (9, 14). Several studies have shown that the SSX2 antigen can activate CD8⁺ T lymphocyte cells to efficiently kill melanoma and carcinoma cells (1, 4). To determine the safety and efficacy of SSX2 as a therapeutic cancer vaccine, sufficient

quantities of it are needed for clinical trials.

In this study, we developed a process suitable for the large-scale production of SSX2 antigen. Coexpressing a pRARE plasmid with the SSX2 production plasmid was first investigated. The production of SSX2 was then scaled up to a 2 L bench-top fermentor operated with a fed-batch protocol to increase cell biomass and SSX2 yield. A purification process that included chromatographic steps including immobilized metal (nickel) affinity, anion exchange and hydrophobic interaction was then developed to improve the purity and quality of SSX2. The purified SSX2 was characterized by its ability to be recognized by anti-SSX2 antibodies from the sera of cancer patients.

Materials and Methods

Bacterial strain and plasmid construction

E. coli C41 (DE3) purchased from Lucigen Corporation (Middleton, WI) was used as the host strain to express recombinant SSX2 (GenBank accession no. **NM175698**). A SSX2 N-terminal six His-tag gene was constructed using PCR by placing Nde1-6His and Not1 fragments at the N and C termini of the SSX2 gene, respectively. The Nde1-NHSSX2-Not1 construct was amplified using the oligonucleotides, 5'-CATATGCACCACCACCACCACCAACGGAGACGACGCC-3' and 5'-GCGGCCGCTTACTCGTCATCTTCCTCAGGGTCGCTG-3', and plasmid pET9a24aSSX2 as a template. The amplified fragment was then cloned into a pCR2.1 plasmid using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) to create plasmid pCR2.1-Nde1-NHSSX2-Not1. The expression plasmid, pET9a24aNHSSX2 (Figure 2-1a) with kanamycin as a selection marker, was constructed by replacing the Nde1-

NotI fragment of vector pET9a24a with the NdeI-NHSSX2-NotI fragment derived from pCR2.1-NdeI-NHSSX2-NotI. The pRARE plasmid (Novagen, Darmstadt, Germany), which encodes tRNAs rarely produced in *E. coli* and uses chloramphenicol as a selection maker, was coexpressed in addition to pET9a24aNHSSX2 to improve SSX2 antigen production.

Cultivation of strain

Shake flasks

A single colony of *E. coli* C41 (DE3) bearing pET9a24aNHSSX2 and pRARE plasmids was inoculated in 5 ml LB medium (1.0% tryptone, 0.5% yeast extract and 0.5% sodium chloride) containing 100 µg/ml kanamycin and 36 µg/ml chloramphenicol. For *E. coli* C41 (DE3) carrying pET9a24aNHSSX2 only, the LB medium did not include chloramphenicol. The cultures were grown overnight at 37°C, and used to inoculate a fresh 30 ml of antibiotic-containing LB medium to an initial optical density (OD₆₀₀) of 0.1. After the culture reached an OD₆₀₀ of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM to induce the production of SSX2. Four hours after induction, the cells were collected, and OD₆₀₀ was normalized to 5.0 to analyze SSX2 production.

Fermentation

An overnight culture of *E. coli* C41 (DE3) bearing pET9a24aNHSSX2 and pRARE was grown in 40 ml of production culture medium (0.1 g/L FeSO₄·7 H₂O, 3 g/L (NaPO₃)₆, 2.1 g/L NH₄Cl, 1.9 g/L citric acid·monohydrate, 2 ml/L 30 % NH₄OH, 15 g/L yeast extract, 20 g/L glucose, 3.2 mM MgSO₄·H₂O, 0.2 mg/L MnCl₂·4H₂O, 100 µg/ml kanamycin and 36 µg/ml chloramphenicol) at 37°C until the OD₆₀₀ reached

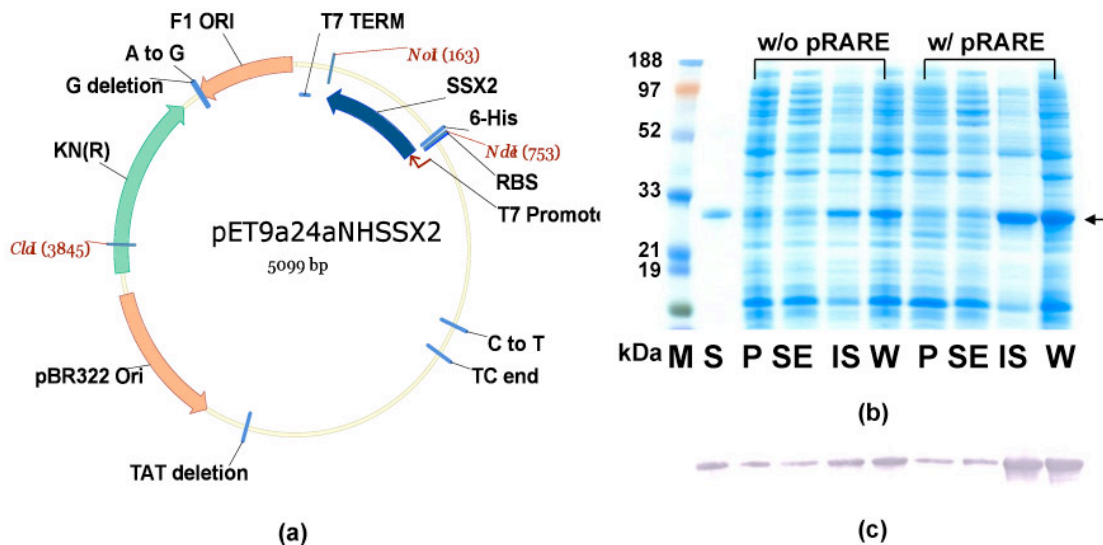


Figure 2-1. Plasmid construction and SSX2 expression. (a) Restriction map of recombinant SSX2 antigen expression plasmid pET9a24aNHSSX2. The 6 His tag gene was cloned at the N-terminus of SSX2 gene. The expression of SSX2 antigen gene was driven by a T-7 promoter and induced by adding IPTG. (b) SDS-PAGE and (c) western blot of SSX2 antigen expression with and without pRARE plasmid in *E. coli* C41 (DE3). Optical density was normalized before analysis. M, molecule weight marker; S, SSX2 standard; P, whole cell lysate before induction; SE, soluble protein fraction after induction; IS, insoluble protein fraction after induction; W, whole cell lysate after induction

2.0. This culture was used to inoculate the fermentor. The fermentation was carried out in a 2 L fermentor (Bioflo 3000, New Brunswick Scientific, Edison, NJ) with an initial volume of 800 ml production culture medium. AFS-Biocommand Bioprocessing software version 2.6 (New Brunswick Scientific) was used for data acquisition and parameter control. During the fermentation, the culture was cultivated at 37°C with agitation at 1000 rpm and an air flow rate of 2.5 L/min. The dissolved oxygen (DO) level was maintained at 40% of air saturation, and the air supply was supplemented with pure oxygen when needed. The pH of the culture medium was maintained at 6.8 with 5 M NaOH. After glucose was depleted from the initial medium, base addition was turned off and the addition of a highly-concentrated glucose feed (275 g/L glucose, 0.1 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.6 g/L sodium citrate-dihydrate, 2.4 mg/L $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$, 3 g/L $(\text{NaPO}_3)_6$, 3 g/L ammonium carbonate, 210 g/L yeast extract, 100 µg/ml kanamycin, 36 µg/ml chloramphenicol and 4 mM IPTG) was initiated. A pH-control feed approach was then used to control the feeding rate. Since IPTG was added to the feed, SSX2 production was induced while the feed was added. Samples were taken for analysis of wet cell weight (WCW) and SSX2 concentration during the fermentation. For WCW and glucose concentration, a 1.0 ml sample of the broth was collected and centrifuged at 13,000 x g for 10 minutes. The supernatant was collected for glucose concentration analysis (Glucose Assay Kit, Sigma-Aldrich, St. Louis, MO) and the cell pellet was weighed to determine WCW.

Purification of recombinant SSX2 antigen

Cell homogenization, inclusion body wash and solubilization

1 L of fermentation broth with 260 g of wet cells was lysed with an EmulsiFlex-C3 microfluidizer (Avestin, Ontario, Canada) for three passes at peak

pressure of 20,000 psi. The cell lysate was then washed with 16 L Tris buffer (50 mM Tris base, 100 mM NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol, pH 7.8) and 8 L Phosphate buffer (100 mM sodium phosphate, 0.5 M NaCl, and 1 mM 2-mercaptoethanol, pH 7.2) using a tangential flow filtration system equipped with a 0.45 μm pore size centramate cassette (Pall, East Hills, NY) that has minimum effective filtration area of 0.09 m^2 . The 500 ml retentate, which contained the inclusion bodies and cell debris, was collected and solubilized in a 3000 ml buffer (8.7 M urea, 100 mM sodium phosphate and 1 mM 2-mercaptoethanol, pH 7.5) to a final urea concentration of 7.5 M.

Immobilized metal (nickel) affinity chromatography (IMAC)

The solubilized protein fractions were filtered with 1.2 and 0.5 μm dead-end membrane filters (Pall) in tandem. The filtrate was then loaded on a 500 ml HiTrap Fast Flow IMAC column (GE Healthcare, Piscataway, NJ) which had been equilibrated with IMAC buffer A (4 M urea, 100 mM sodium phosphate and 1 mM 2-mercaptoethanol, pH 7.5). The chromatographic separation was carried out using an AKTA Explorer (GE Healthcare). After solubilized protein fractions were applied to the IMAC column, the column was washed with 5% IMAC buffer B (4 M urea, 100 mM sodium phosphate, 500 mM imidazole and 1 mM 2-mercaptoethanol, pH 7.5), and SSX2 was then eluted with 100% IMAC elution buffer B. The eluted fraction containing SSX2 was buffer exchanged (4 M urea, 50 mM Tris and 1 mM 2-mercaptoethanol, pH 8.0) and concentrated to 450 ml by a tangential flow filtration system with a 10 kDa cut-off centramate cassette (Pall) that has minimum effective filtration area of 0.09 m^2 .

Anion-exchange chromatography

Anion-exchange chromatography was performed by using a 5 ml Q Sepharose High Performance (Q HP) column (GE Healthcare) which had been equilibrated with AEX buffer A (4 M urea, 50 mM Tris and 1 mM 2-mercaptoethanol, pH 8.0). A total of 6 ml of the buffer-exchanged SSX2 fraction from IMAC was applied to the Q HP column. SSX2 was mainly eluted during a step gradient of 40% AEX buffer B (4 M urea, 50 mM Tris, 1 M NaCl and 1 mM 2-mercaptoethanol, pH 8.0). Fractions 3 to 5 (Figure 2-5) were collected and pooled for the next purification step.

Hydrophobic interaction chromatography

Anion exchange elution fractions containing SSX2 were mixed with an equal volume of pre-HIC addition buffer (4 M urea, 50 mM Tris, 1.4 M ammonium sulfate and 1 mM 2-mercaptoethanol, pH 8.0). A total of 30 ml of this solution was loaded onto a 5 ml Phenyl-Sepharose High Performance (Phenyl HP) column (GE Healthcare) which was equilibrated with HIC buffer B (4 M urea, 50 mM Tris, 1 M ammonium sulfate and 1 mM 2-mercaptoethanol, pH 8.0). SSX2 was then eluted in a step gradient of 50% HIC buffer A (4 M urea, 50 mM Tris and 1 mM 2-mercaptoethanol, pH 8.0).

SDS-PAGE and immunoblotting

Samples were analyzed for SSX2 by electrophoresis on 12% SDS-polyacrylamide gels (Invitrogen). After electrophoresis, the gels were stained by SimplyBlue SafeStain (Invitrogen). For western blot analysis, the proteins were transferred to a nitrocellulose membrane by electrophoresis and SSX2 was detected with 2.5 µg of murine anti-human SSX2 monoclonal antibody (24) using a WesternBreeze kit (Invitrogen). All assays were performed according to

manufacturer's instructions.

Protein concentration

Total protein concentration was determined by a Coomassie (Bradford) protein assay kit (Pierce Biotechnology, Rockford, IL) using bovine serum albumin protein as a standard. SSX2 concentrations were estimated from scanned images of the developed western with Image J densitometry software (<http://rsb.info.nih.gov/ij>). Western blots were scanned and images with SSX2 protein bands were selected, plotted and compared with an SSX2 standard. The purity of SSX2 was determined by estimating the total amount of SSX2 as compared to the total amount of protein in each sample.

Endotoxin concentration

Endotoxin in different chromatographic preparations were quantified by using a Limulus Amebocyte Lysate (LAL) Endosafe Endochrome-K kit (Charles River Laboratories, Wilmington, MA) according to the manufacturer's instructions. Samples were diluted in LAL Reagent water and 100 μ l aliquots were added to a 96 well plate before the addition of 100 μ l of a chromogenic substrate. The kinetic colorimetric data of samples were collected by TECAN GENios microplate reader (TECAN Group Inc., Mannedorf, Switzerland). All assays were performed in triplicate and the average value was presented.

Enzyme-link immunosorbent assays

10 μ l/well of 1 μ g/ml purified recombinant SSX2 in a coating buffer (15 mM Na_2CO_3 , 30 mM NaHCO_3 and 0.02% NaN_3 , pH 9.6) was added to a well of TC microwell plates 60 \times 10 (Nunc, Roskilde, Denmark) and incubated overnight at 4°C.

Plates were washed with phosphate buffered saline (PBS) and blocked overnight at 4°C with 10 µl/well of 2% BSA/PBS. Then 10 µl/well of serum diluted in 2 % BSA was added and incubated for 2 h at room temperature. Plates were washed again, and 10 µl/well of diluted secondary antibody (Goat anti-human IgG-AP, Southern Biotechnology, Birmingham, AL) with 2% of BSA was added and incubated for 1 h at room temperature. Plates were washed, incubated with 10 µl/well of Attophos substrate solution (JBL Scientific, Obispo, CA) for 25 minutes at room temperature. Fluorescence was immediately read by a plate reader (CytoFluor 2350, Millipore, Bedford, MA) at the wavelength of 580nm.

Results

Effect of pRARE on expression of SSX2

E. coli C41 (DE3) bearing the pET9a24aNHSSX2 plasmid was induced and the soluble and insoluble protein fractions were analyzed. SSX2 was produced at the concentration of 13.9 mg/ml and accumulated mainly as inclusion bodies (Figure 2-1b and 2-1c). In order to increase SSX2 expression, we coexpressed pET9a24aNHSSX2 with a pRARE plasmid, which codes for rarely used tRNA in *E. coli*. The production of SSX2 antigen in cell cultures with both plasmids was 22.4 mg/ml, a 1.6 fold higher production than *E. coli* cultures bearing pET9a24aNHSSX2 only (Figure 2-1b and 2-1c). Because of its potential for producing a higher amount of SSX2 antigen, the transformant carrying both pET9a24aNHSSX2 and pRARE plasmids was used to scale up in a 2 L fermentor.

Large scale production of SSX2 in 2 L fermentor

The production profile of SSX2 is illustrated as Figure 2-2. At 5.5 hours of cultivation, feeding was initiated, and the production of SSX2 was induced. After

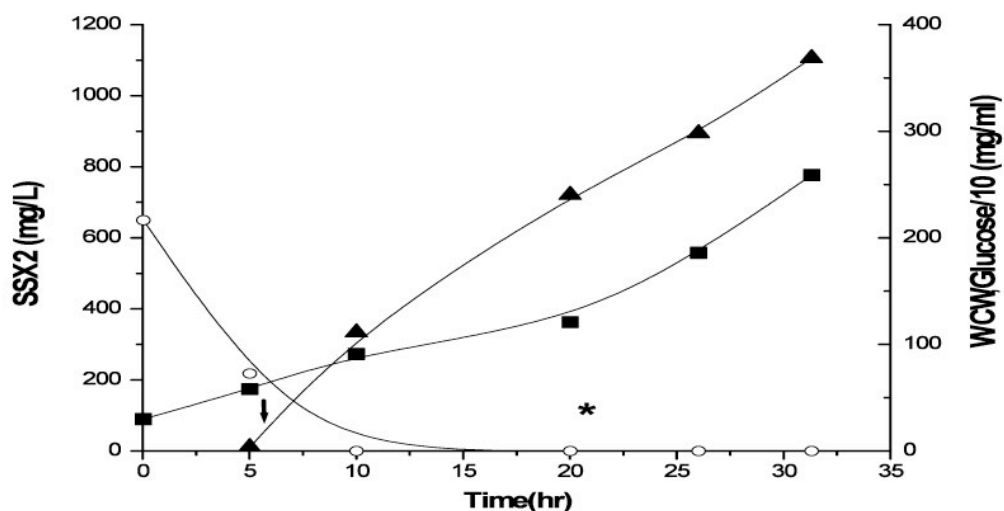


Figure 2-2. Production profile of SSX2 in *E. coli* C41 (DE3) harboring pET9a24aNHSSX2 and pRARE plasmids cultivated at 37°C using 2 L fermentor. The arrow indicates the time of feed initiation. The asterisk indicates the feed of oxygen. ▲, SSX2 concentration; ■, wet cell weight; ○, glucose concentration; WCW, wet cell weight

Table 2-1. Total protein, SSX2 concentration and percent recovery in preliminary steps of the purification process from cell lysis through IMAC elution

Fractions	Total Protein (mg)	SSX2 (mg)	Recovery (%)
After microfluidizer	16987	1184	100
Tris wash retentate	7310	1063	90
Tris wash permeate	11744	0	-
Phosphate wash retentate	7285	919	78
Phosphate wash permeate	354	15	-
After solubilization	6720	889	75
IMAC flow through	3675	302	-
IMAC wash	1470	229	-
IMAC elution	1170	403	34

induction, glucose concentration was maintained below 0.1 g/L. At 21 hrs of cultivation, inlet air was supplemented with oxygen to maintain a dissolved oxygen level of 40% air saturation. During the fermentation, the amount of cell biomass and the quantity of SSX2 increased steadily throughout the induction period at a rate of 6.7 mg/ml/hr and 39 mg/L/hr, respectively. After cultivation for 32 hrs and 1.2 L feed had been added, the cell biomass reached 260 mg/ml and approximately 1.1 g/L of SSX2 antigen had been produced.

Purification of SSX2

In the large scale fermentation, SSX2 was also mainly accumulated in the insoluble fraction (Figure 2-3). Therefore, after cells were lysed, the inclusion bodies were washed and soluble proteins were discarded. During the wash, roughly 12 g or 71% of total protein (as measured by Bradford) was removed by TFF with limited SSX2 antigen loss in the permeate (Figure 2-3 and Table 2-1). The retentate was solubilized and applied to the IMAC column. In this purification step, the flowthrough and low imidazole steps removed a fair amount of protein impurities with 531 mg or 60% loss of SSX2. However, the purity of SSX2 in the high imidazole elution was improved from 13% to 34% (Table 2-1 and Figure 2-4). The chromatogram and SDS-PAGE of eluted fractions from anion exchange are shown in Figure 2-5. SSX2 was mostly eluted at 400 mM of NaCl and its purity was further improved to 43% (Table 2-2). The 3.44 mg of eluted SSX2 was then loaded on a third chromatography column, a 5 ml Phenyl HP column, which utilized hydrophobic interaction. SSX2 was mainly eluted at an ammonium sulfate concentration of 500 mM, while the high molecular weight protein impurities eluted at an ammonium sulfate concentration below 100 mM (Figure 2-6). The SSX2 product from the entire purification process was over 87% pure and the yield was 20% (Table 2-2). Based on

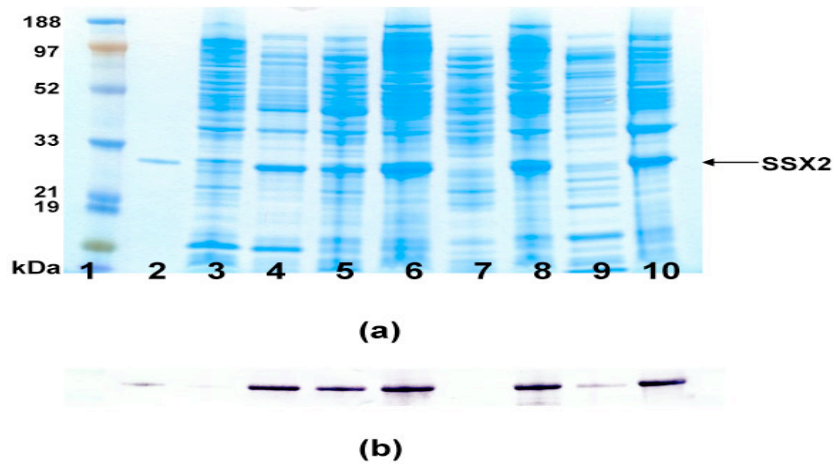


Figure 2-3. Coomassie-stained SDS-PAGE (a) and western blot (b) of SSX2 antigen fractions prior to chromatography. Lanes: 1, molecular weight marker; 2, SSX2 standard; 3, soluble protein fraction of cell lysate; 4, insoluble protein fraction of cell lysate; 5, total cell lysate after microfluidizer; 6, Tris wash retentate; 7, Tris wash permeate; 8, phosphate wash retentate; 9, phosphate wash permeate; 10, 7.5 M urea solubilized fraction. Samples of lane 7 and 9 were concentrated before loading on the gel

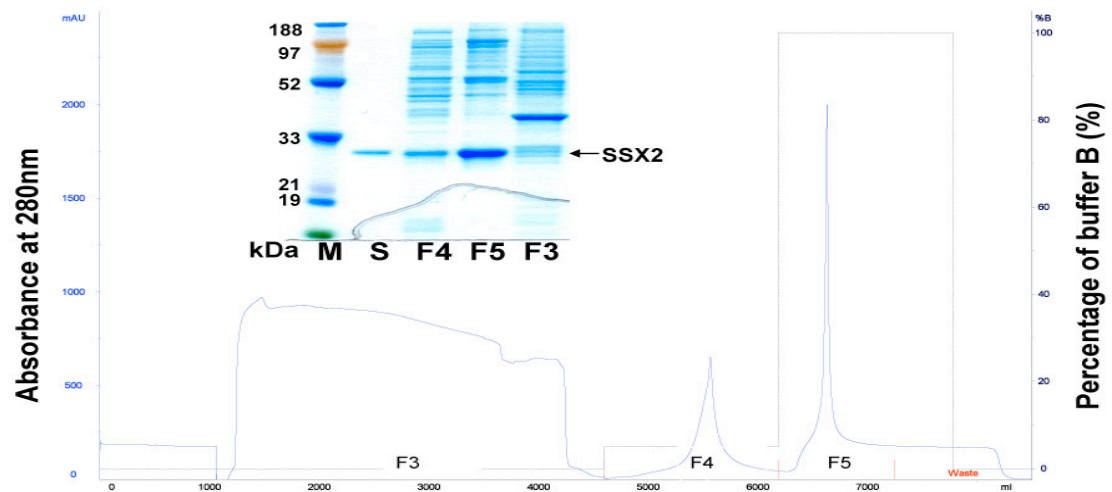


Figure 2-4. FPLC loading and elution profile of solubilized SSX2 antigen applied on 500 ml immobilized metal (nickel) affinity chromatography (IMAC, Ni-NTA) column. F3, flow through fraction; F4, wash fraction; F5, elution fraction. Blue line indicates absorbance at 280 nm and gray line represents percentage of IMAC B buffer. Inset: Coomassie-stained SDS-PAGE of fractions from IMAC chromatography. M, molecular weight marker; S, SSX2 standard

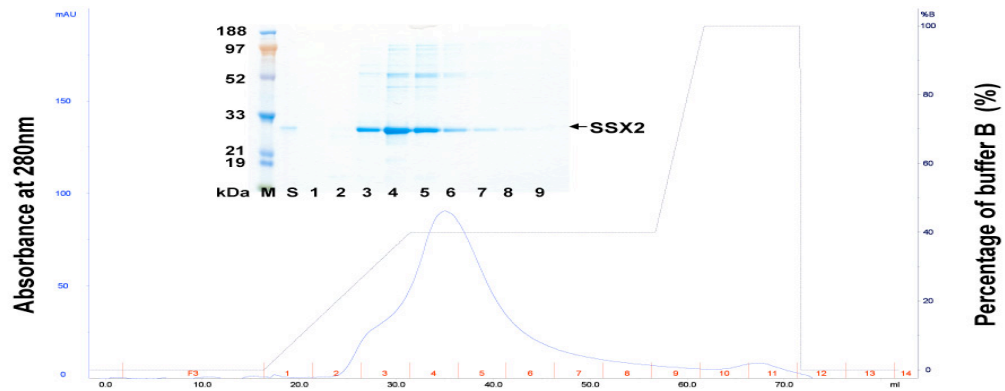


Figure 2-5. Anion exchange chromatography profile of 6 ml buffer-exchanged high imidazole fraction on 5 ml Q Sepharose HP column at pH 8. SSX2 antigen was mainly eluted at 400 mM of NaCl. Blue line indicates absorbance at 280 nm and gray line represents percentage of anion exchange B buffer. Fractions 3 to 5 were collected and pooled for HIC process. Inset: Coomassie-stained SDS-PAGE of fractions derived from anion exchange chromatography. Lane numbers correspond to chromatogram fraction numbers. M, molecular weight marker; S, SSX2 antigen standard

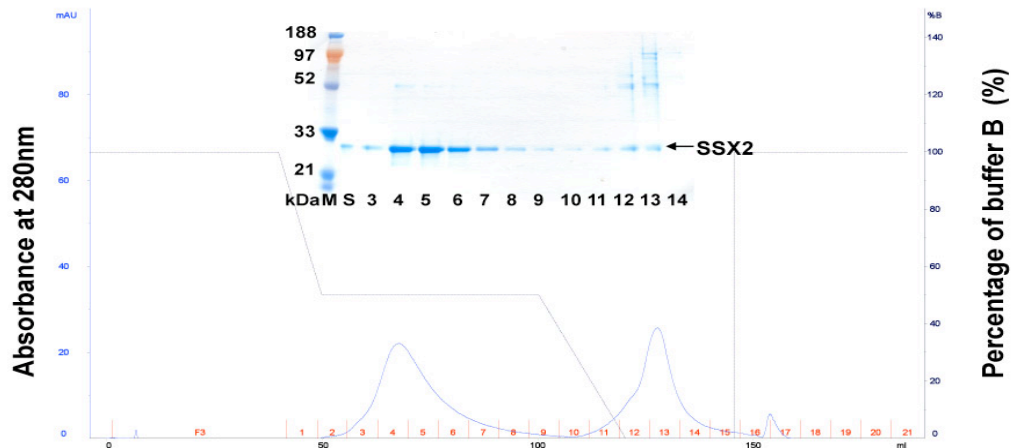


Figure 2-6. Hydrophobic interaction chromatography (HIC) profile of SSX2 antigen peak fractions from Q Sepharose HP column on a 5 ml Phenyl-Sepharose HP column. SSX2 antigen was mainly eluted at 0.5 M ammonium sulfate. Blue line indicates the absorbance at 280 nm and gray line represents percentage of HIC buffer B. Inset: Coomassie-stained SDS-PAGE of fractions derived from hydrophobic interaction chromatography. Fractions 3 to 7 were collected as final purified SSX2 antigen. Lane numbers correspond to chromatogram fraction numbers M, molecular weight marker; S, SSX2 antigen standard

the current scale, it is estimated that a total of 236.3 mg of SSX2 can be produced per liter of fermentation.

Endotoxin assay

Samples were collected at each stage of the purification process and analyzed for endotoxin content (Table 2-2). The endotoxin content of the buffer-exchanged fraction obtained after IMAC was 107 EU/ μ g of total protein. Anion exchange chromatography further decreased endotoxin nearly five-fold to 23 EU/ μ g of total protein. After hydrophobic interaction chromatography, SSX2 antigen elution fractions had an endotoxin level of 0.11 EU/ μ g of total protein, a 209-fold reduction.

Immunological reaction of SSX2 with human sera

Sera from eight cancer patients, some of whom had anti-SSX2 antibodies, were tested for reactivity to purified recombinant SSX2 (Table 2-3). All three sera that have been previously shown to have anti-SSX2 antibodies reacted with the recombinant SSX2 and the titer of the sera against the recombinant SSX2 correlated with known antibody activities. Patients' sera that had been reported to not contain anti-SSX2 antibodies did not react with the purified SSX2. Together, these results indicate that SSX2 produced in this study was able to be specifically recognized by anti-SSX2 antibodies from the sera of cancer patients.

Discussion

It has been shown in several studies that coexpression of a pRARE plasmid can improve recombinant protein expression and solubility in the cytoplasm due to the increased supply of rare tRNAs for *E. coli in situ* (5, 11). In this study, we also found that coexpression of SSX2 with pRARE plasmid increased SSX2 production

Table 2-2. Total protein, SSX2 antigen concentration, percent recovery and endotoxin content of anion exchange and hydrophobic interaction chromatography

Fractions	Total Protein (mg)	SSX2 (mg)	Recovery (%)	Endotoxin (EU/ug)
After buffer exchange from IMAC	14.4	5.28	33	107±26.8
Post anion exchange	7.8	3.44	22	23±1.4
Post hydrophobic interaction	3.6	3.15	20	0.11±0.04

Table 2-3. ELISA activity of selected human cancer serum with SSX2 antigen purified from this study

Sera from different cancer patients	Anti-SSX2 antibody activity ¹	Measured ELISA titer	
		SSX2 ²	<i>E. coli</i> lysate
Lung Cancer-1	Moderate Strong	1:3200	Negative
Lung Cancer-2	Weak	1:800	Negative
Lung Cancer-3	Negative	Negative	Negative
Lung Cancer-4	Negative	Negative	Negative
Lung Cancer-5	Negative	Negative	Negative
Lung Cancer-6	Negative	Negative	Negative
Breast Cancer-1	Moderate Strong	1:3200	Negative
Neuroblastoma-1	Negative	Negative	Negative

¹: The anti-SSX2 antibody activity of the sera from cancer patients (unpublished data).

²: SSX2 antigen purified from this study.

1.6-fold when the number of cells was normalized. In order to further increase SSX2 production, a 2 L fermentation incorporating a fed-batch strategy was used to achieve high cell density and SSX2 productivity. Studies have showed that a fed-batch production scheme has advantages for the production of recombinant proteins in *E. coli* (22). Normally high production rates will lead to acetic acid accumulation due to an insufficient oxygen supply or the presence of excess glucose in the medium. Accordingly, it is crucial to control the DO level and glucose concentration during a fermentation (7). It is reported that feeding strategies using DO or pH as an indicator can prevent acetic acid accumulation and promote recombinant protein production (12). Therefore, in this study, the DO level was maintained over 40% of air saturation by supplying oxygen into the fermentor when agitation and sparging with air only would not have been able to satisfy oxygen demand. Additionally, a pH-controlled feed approach was used to optimize the glucose supply. In such a process, cells are kept near glucose starvation by using pH to control the glucose feeding rate (13). IPTG was added to the feed medium instead of being added to the fermentor in a single dose. This strategy is based on the idea that the concentration of IPTG in the fermentation will increase with cell mass ensuring it is at a concentration sufficient for induction. Together these strategies provide steady increase in both cell biomass and SSX2 allowing cell biomass and SSX2 produced to reach 260 mg/ml and 1105 mg/L respectively at the end of the fermentation.

Since SSX2 was produced mainly as inclusion bodies necessitating their concentration, our purification process targeted the insoluble protein fraction. Centrifugation and TFF are the two most common methods used to collect inclusion bodies (3, 21). Our previous work has shown that protein loss is minimized when TFF is used instead of centrifugation. Additionally, scale-up of centrifugation can be

difficult (10), hence we focused on TFF. In this study, our data also showed that TFF washes effectively retained approximately 78% of SSX2 (Table 2-1).

The addition of His-tags aids in the purification of a protein and is not an impediment to the use of recombinant proteins in human clinical trials (9, 15). Approximately 60% of SSX2 was lost in the flowthrough and wash fractions from the IMAC chromatography. We found that loss of SSX2 in the flowthrough was not a result of exceeding the binding capacity of this IMAC column; a western blot of the flowthrough fractions showed a similar concentration of SSX2 in all fractions (data not show). The composition and conformation of a protein dictates the binding affinity in IMAC (28). The inability of the majority of the SSX2 to bind to the IMAC may be the result of aggregation that occludes the His-tag and prevents it from binding to the Ni-NTA groups.

Anion exchange chromatography was used to further purify the SSX2. Although the anion exchange did not significantly remove host proteins contaminants as compared to IMAC and HIC, it did reduce endotoxin content five-fold. It has been reported that anion exchange chromatography can remove endotoxin from *E. coli*-derived recombinant proteins (25). Endotoxin in recombinant biopharmaceuticals can cause a variety of acute inflammatory responses in humans, and endotoxin levels are an important criteria for the quality of a biopharmaceutical (17). Since this process is developed with the intention of using SSX2 in human clinical trials, removing endotoxin through anion exchange chromatography was deemed to be an integral part of the purification process.

Hydrophobic interaction chromatography (HIC) not only improved the purity of SSX2, but it reduced the endotoxin level 209-fold to 0.11 EU/ μ g of total protein. Due to its predicted high hydrophilicity, it is not surprising that SSX2 antigen eluted at

a higher ammonium sulfate concentration than the high molecule weight impurities which eluted toward the end of the chromatography. Several studies have demonstrated that endotoxin will bind to the HIC matrix (23, 27) and elute at the end of the gradient due to its hydrophobic nature (data not show). This also indicates that the HIC process is crucial for acquiring pure SSX2 antigen with low endotoxin levels. Finally, in this study, we also performed an ELISA assay to characterize the purified SSX2 antigen. CT antigens including SSX2 are known to be expressed heterogeneously in cancer patients (6). The SSX2 antigen prepared in this study retained the functionality of being able to be recognized specifically by sera of cancer patients who have anti-SSX2 antibodies. In summary, we reported a production and purification process to produce the SSX2 antigen with reasonable yield, high purity and low endotoxin content that should be sufficient for further scale up.

Acknowledgements

The authors thank colleagues at the Cornell University/Ludwig Institute for Cancer Research Partnership for the invaluable discussions and suggestions. This work was supported by the Ministry of Education, Taiwan and the Ludwig Institute for Cancer Research.

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CHAPTER 3

PROCESS DEVELOPMENT IN SSX2 CANCER VACCINE PREPARATION FOR CGMP PRODUCTION

Abstract

SSX2 is a Cancer Testis antigen expressed in a wide variety of cancers including synovial sarcoma and melanoma. Humoral and cellular immune responses against SSX2 gene products have been described making SSX2 an attractive vaccine target for cancer immunotherapy. For the purpose of human Phase I/II proof-of-principle clinical trials, SSX2 has been expressed in *E. coli* C41(DE3) and produced in a 2 L scale of fermentation and purification. Further process developments were made to a previously reported process to improve its production yield, endotoxin level and purity. The yield and purity from anion exchange chromatography were enhanced by changing the buffer from Tris to phosphate. A secondary anion exchange chromatography was introduced later in the process specifically for endotoxin removal. This process decreased the endotoxin level from 4300 EU/mg to 20 EU/mg, a 215-fold reduction. A 10 L scale development run for SSX2 production at the Cornell/LICR cGMP Bioprocess Facility, which produced 420 mg of SSX2 with endotoxin level less than 40 EU/mg and purity over 97%, is also presented. The purified urea-solubilized SSX2 was refolded on metal affinity chromatography with a yield of 80%. The secondary structure of refolded SSX2 was characterized by Circular Dichroism and approximated that it is comprised of 23% alpha helix, 19% of beta sheet and 58% of random coil.

Huang C-J., Newton, S. B., Chen, H. R., Anderson, A. K., Damasceno, M. L., Ritter, G., Old, . L., Batt A. C. Process development for SSX2 cancer vaccine preparation for cGMP production. Manuscript to be submitted, Cornell University.

Introduction

Cancer is a group of diseases caused by the growth and spreading of abnormal cells or cancer cells. It is one of the top life-threatening diseases in the United States: a total of over 1.4 million new cancer cases and half million of deaths is projected in 2008 (9). Although the treatments for cancers, including surgery, radiotherapy, chemotherapy and hormone therapy, have been established and combinations of these therapies have been applied, cancer still causes 25% of mortalities. Therefore, immunotherapeutic approaches to treat cancers or cancer immunotherapy have developed in recent years as an alternative modality of cancer treatment. Unlike other therapies to eradicate tumor cells by radiation or drugs, cancer immunotherapy, which includes passive and active immunotherapy, aims to bolster a patient's immune system to destroy existing cancer cells (14). Passive immunotherapy uses tumor specific monoclonal antibodies to target tumor cells to attack tumor cells or block the factors that enable a cancer to grow or to spread (18). Active immunotherapy, however, relies on the administration of a cancer vaccine, a tumor-specific antigen or peptide, to generate anti-cancer immune responses. It aims at raising both cellular and humoral immune responses against cancer cells to kill cancer cells (10).

Cancer-Testis (CT) antigens are one class of these tumor specific antigens that are an expanding family of targets for active cancer immunotherapy. CT antigens are highly tumor-specific: the genes encoding CT antigens are expressed in different tumors, but they are not expressed by normal tissues with exception of testis germline cells. Fortunately, germline cells are MHC class I negative, thus not recognize by cytotoxic T lymphocytes, making CT antigens ideal targets for cancer immunotherapy (13). Today, the clinical trails of CT antigens, MAGE A3 and NY-ESO-1, as cancer vaccines have shown good results (6, 10), suggesting that active immunotherapy is a

promising treatment for cancers.

Synovial sarcoma X break point 2 (SSX2), belongs to a highly homologous family that encodes proteins involved in a chromosomal translocation t(X;18)(p11.2;11.2) characteristically found in 70% of human synovial sarcoma (4). It is also known as HOM-MEL-40, a CT antigen identified from a melanoma tumor, that is also expressed in different malignancies, including colon cancers, hepatocarcinoma, and breast carcinoma (17). Both cellular and humoral immune responses against SSX2 antigen have been described in literatures, making SSX2 an attractive cancer vaccine candidate for active cancer immunotherapy (2, 13). In contrast to conventional oncology drug development which is designed for cytotoxic agents, cancer vaccines require more flexible and developmental guidelines to address their therapeutic effects. In 2006, Cancer Vaccine Clinical Trial Working Group (CVCTWG) proposed a clinical development paradigm for cancer vaccines and related biologics (7). It suggested that a proof-of-principle trial should be introduced in advance to a minimum of 20 patients to establish safety database and to demonstrate biological activity of cancer vaccine candidates. Therefore, sufficient quantities of potential cancer vaccines must be produced under current Good Manufacturing Practice (cGMP) guidelines beforehand to perform this proof-of-principle trial.

To evaluate the efficacy of SSX2 as a cancer vaccine, we previously reported the production and purification of SSX2 from *Escherichia coli* C41(DE3) in a 2 L bench-top fermentor (8). In the present study, changes to the previous process have been explored to improve protein yield and quality. The result of a development run of the new process at Cornell/LICR cGMP biologics is included. Finally, urea-solubilized SSX2 was refolded, and was characterized by Circular Dichroism. From this study, we aim to provide information necessary to produce sufficient amount and

high quality of recombinant SSX2 for cancer vaccine trials.

Materials and methods

Strain construction and preparation of recombinant SSX2

The construction of the expression strain and preparation of SSX2 protein were as previously described (8) with slightly modifications. In brief, the full length of SSX2 (Genbank accession No. NM175698) was tagged with six histidine residues on its N-terminus and cloned into the plasmid pET9a24a to create expression plasmid pET9a24aNHSSX2. *E. coli* C41(DE3) bearing pET9a24aNHSSX2 and pRARE plasmid, which encodes tRNAs rarely used in *E.coli*, was used as a SSX2 production strain. A single colony of it was inoculated in 5 ml LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) and grew at 37°C overnight before being transferred to 40 ml of fermentation medium (0.1 g/L FeSO₄·7H₂O, 3 g/L (NaPO₃)₆, 2.1 g/L NH₄Cl, 1.9 g/L citric acid·H₂O, 2 ml/L 30 % NH₄OH, 15 g/L yeast extract, 20 g/L glucose, 3.2 mM MgSO₄·7H₂O, 0.2 mg/L MnCl₂·4H₂O, 100 µg/ml kanamycin and 36 µg/ml chloramphenicol) to allow the optical density (OD₆₀₀) to reach 2.0. This culture was used to inoculate a 2 L fermentor (Bioflo 3000, New Brunswick Scientific, Edison, NJ) with an initial volume of 800 ml of fermentation medium connected with a fermentation feed (275 g/L glucose, 0.1 g/L FeSO₄·7H₂O, 10.5 g/L MgSO₄·7H₂O, 2.6 g/L sodium citrate·2H₂O, 2.4 mg/L MnCl₂·4H₂O, 3 g/L (NaPO₃)₆, 3 g/L ammonium carbonate, 210 g/L yeast extract, 100 µg/ml kanamycin, 36 µg/ml chloramphenicol and 40 mM IPTG). At the end of the batch phase, a fed-batch fermentation was initiated by a pH-state control until the end of the fermentation. The cells were harvested, centrifuged and lysed, and the SSX2 inclusion bodies were washed then

solubilized in 7.5 M urea as previously described (8). The solubilized SSX2 was loaded on a 500 ml immobilized metal chelating chromatography (IMAC) column (Chelating Sepharose Fast Flow, GE Healthcare, Piscataway, NJ), and the eluted SSX2 was subjected to a buffer exchange with Tris buffer A (4 M urea, 50 mM Tris, pH=7.5) or AEX buffer A (4 M urea, 50 mM phosphate, pH=7.5). All chromatographic separation was carried out using AKTA explorer (GE healthcare).

Anion exchange chromatography and endotoxin removal

A total of 120 mg of buffer exchanged SSX2 in Tris buffer A was loaded on 150 ml of Q sepharose High Performance resin (QHP, GE Healthcare). SSX2 was eluted at a step gradient of 30% Tris buffer B (4M urea, 50mM Tris, 1 M NaCl, pH=7.5). Eluted fractions were collected and pooled for analysis. A similar experiment was performed by using the phosphate buffer, and SSX2 was also eluted at a step gradient of 30% AEX buffer B (4 M urea, 50 mM phosphate, 1 M NaCl, pH=7.5) for 6 column volumes. The SSX2 eluted using the phosphate buffer was mixed with an equal volume of HIC loading buffer (4 M urea, 50 mM phosphate, 2M ammonium sulfate, pH=7.5) and loaded on a 50 ml Phenyl High Performance (Phenyl HP, GE Healthcare) column that had been equilibrated with HIC buffer A (4 M urea, 50 mM phosphate, 1 M ammonium phosphate, pH=7.5). SSX2 was then eluted at a linear gradient at 50% AEX buffer A. The pooled SSX2 fractions were then buffer exchange with AEX buffer A and this material was used to study endotoxin removal by QHP and Q sepharose XL (QXL, GE Healthcare) columns. A total of 10 mg of this buffer exchanged SSX2 was loaded on 5 ml QHP and QXL columns, respectively, and followed by eluting at a step gradient of 30% AEX buffer B. The endotoxin levels and protein concentrations in the flow through and eluted fractions were collected and analyzed.

Production and purification of SSX2 in cGMP facility

Fermentation and midstream processing

A 0.5 ml of glycerol stock of the SSX2 production strain was inoculated in a 40 ml of fermentation medium and grown overnight at 37°C. This culture was inoculated in a 300 ml of fermentation medium for 2 to 3 hrs until the OD₆₀₀ reached 2.0 and, this was used as inoculum of fermentor. The fermentation was carried out in a New Brunswick Scientific BioFlo 4500 bioreactor with an initial volume of 7 L fermentation medium. The pH, temperature, D.O. controls and feed strategy were as previously described (8). A 9.0 L fermentation feed was prepared and added into the fermentor during the fed-batch phase until the end of the fermentation. Cells were centrifuged, and collected cell pellets were disrupted by a microfluidizer (Microfluidics, Newton, MA) at a pressure of over 20,000 psi. The SSX2 inclusion bodies were washed with Tris (50 mM Tris, 100 mM NaCl and 1mM EDTA, pH7.8) and phosphate buffer (100 mM sodium phosphate and 0.5M NaCl pH 7.2) by tangential flow filtration system equipped with a 0.2 µm pore size centramate cassette (Pall East Hill, NY). After wash, SSX2 was concentrated to 500 ml and solubilized for 4 hrs at a final urea concentration of 7.5 M.

Chromatographic purification of SSX2

Immobilized metal cheating chromatography (IMAC)

Solubilized SSX2 was applied to a 4500 mL Chelating Sepharose Fast Flow IMAC column, and washed with 5% IMAC buffer B (4 M urea, 50 mM phosphate, 500 mM imidazole, pH=7.5). SSX2 was then eluted with 100% IMAC buffer B and buffer exchanged with AEX buffer A by tangential flow filtration system equipped with a 10 kDa cut off pore size centramate cassette (Pall).

QHP anion exchange chromatography

The buffer exchanged SSX2 was loaded on a 3000 mL Q sepharose HP column which had been equilibrated with AEX buffer A. Elution of SSX2 was performed at a step gradient of 30% AEX Buffer B as indicated previously. The SSX2 elute was buffer-exchanged again with AEX buffer A by tangential flow filtration system equipped with a 10 kDa cut off pore size centramate cassette (Pall).

Hydrophobic interaction chromatography

Buffer exchanged SSX2 was mixed with an equal volume of HIC loading buffer and load on the 2500 ml Phenyl HP column that have been equilibrated with HIC buffer A. SSX2 was then eluted at a linear gradient with of 40 to 50% of HIC buffer A. the pooled SSX2 fraction was buffer exchanged again with AEX buffer A as previously described.

QXL anion exchange chromatography

SSX2 was then loaded on 1000 ml QXL column equilibrated with AXE buffer A and chased with two column volumes AEX buffer A. The flow through fraction, where the majority of SSX2 was located, was collected for analysis.

Refolding of SSX2 on IMAC

A total of 5 mg of purified SSX2 was loaded onto a 5 ml IMAC column. The bound protein was refolded using a linear gradient of AEX buffer A and B buffer (50 mM phosphate, pH=7.5). After an additional three column volumes wash of B buffer, the refolded SSX2 was eluted with C buffer (50 mM phosphate, 0.5 M imidazole, pH=7.5). To remove the salts, the column elution was buffer exchanged into 10 mM phosphate (pH 7.5) buffer and analyzed by circular dichroism.

SDS-PAGE and immunoblotting

Protein samples containing SSX2 were analyzed on 10 or 12% SDS polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. Gels were stained by SimpleBlue Safe stain (Invitrogen, Carlsbad, CA) for an hour and then detained in water for 16 hrs. For western blot analysis, proteins were electroblotted onto a nitrocellulose membrane and SSX2 was detected with 2.5 µg of murine anti-SSX2 antibody using a WesternBreeze kit (Invitrogen) according to manufacturer's instructions.

Protein concentration

Total protein concentration was determined by a Coomassie (Bradford) protein assay kit (Pierce Biotechnology, Rockford, IL) by using different concentrations of bovine serum albumin (BSA) protein as standards. 10 µL of different protein samples were added to a 96 well plate, and, 295 µL of Coomassie reagent was added to each well. After incubation at room temperature for 10 mins, the absorbances of different samples at 595 nm were collected by a TECAN GENios microplate reader (TECAN Group Inc., Mannedorf, Switzerland). Protein concentration of each sample was determined by the BSA standard curve. SSX2 purity was estimated from a scanned image of the final bulk SSX2 protein from a coomassie gel with Image J densitometry software (<http://rsb.info.nih.gov/ij>). The lane was scanned, plotted and compared SSX2 with other proteins in the lane to determine its purity.

Endotoxin assay

Endotoxin in different protein samples was quantified by using a Limulus Amebocyte Lysate (LAL) Endosafe Endochrome-K kit (Charles River Laboratories, Wilmington, MA). Samples were diluted in LAL Reagent water and 100 µL aliquots

were added to a 96 well plate before the addition of 100 μ L of a chromogenic substrate according to manufacturer's instructions. The kinetic colorimetric data of samples were collected by TECAN GENios microplate reader (TECAN Group Inc.,). All assays were performed in triplicate and the average value was presented.

Circular Dichroism (CD) Spectrometry and protein secondary structure predictions

CD spectra of refolded SSX2 was obtained with an Aviv 400 Circular Dichroism Spectrometer (Aviv Biomedical, Lakewood, NJ). Protein concentration of 0.05 mg/ml of SSX2 in 10 mM phosphate buffer was scanned by far-UV (190 to 260 nm) wavelength at 25°C. The data from three independent runs were corrected for CD signals from the buffer and averaged. For deconvolution of CD spectra, the original data were analyzed by the *k2d* software (<http://www.embl-heidelberg.de/%7Eandrade/k2d.html>) to predict the secondary structure of refolded SSX2. The predicted secondary structures from primary SSX2 sequence was carried out on PredictProtein server (12)

Results and discussion

Improved yield by using phosphate buffer in anion exchange chromatography

In the previous process, the anion exchange chromatography had shown that even though the endotoxin decreased by five-fold, the protein yield was 54% with limited improvement in protein purity (8). In order to improve the recovery yield, phosphate buffer was used and compared its protein yield with original Tris buffer. As

indicated in Table 3-1, the total protein recovery in SSX2 elution fractions was 67% in phosphate buffer compared to 51% in Tris buffer. The total protein recovery for Phosphate and Tris buffer were 85% and 55%, respectively. FPLC chromatograms and gels of different fractions from two anion exchange processes were shown in the Figure 3-1. Unlike Tris buffer, host proteins were removed in the flow through by Phosphate buffer. These results suggested that, in Tris buffer, some proteins, including SSX2 and host proteins, were bound to the resin even after the 1 M NaCl wash, leading to low protein recoveries in both peak fractions and total protein content. This might be caused by the lower solubility of SSX2 protein solution in 4M urea Tris buffer indicated by cloudiness after buffer exchange from IMAC elute (data not shown). Since the phosphate buffer improved protein yield in the anion exchange, it was used throughout the purification process.

Endotoxin removal by Q Sepharose XL

It is known that endotoxin can be removed from protein solution by anion exchange chromatography due to its negatively charged property. However, this method is generally applied to remove endotoxin from basic proteins which are repelled by anion-exchanger (11). Since SSX2 is an acidic protein ($pI=6.2$), the clearance factor of endotoxin is limited to around three to five fold by using Q Sepharose HP (QHP) resin. To effectively reduce endotoxin, a similar but bigger bead size resin, Q Sepharose XL (QXL), was tested. As shown in Figure 3-2 (A and B), unlike QHP, 65% of SSX2 was found in the flow through fraction of QXL and its endotoxin was at the concentration of 20 EU/mg (Figure 3-2 D). It decreased the endotoxin level from 4300 EU/mg to 20 EU/mg, a 215-fold reduction. In addition, the the SSX2 found in the eluted fraction contained higher molecular weight impurities,

Table 3-1. Comparison of protein recovery of Tris and Phosphate buffer in anion exchange process

Buffers	Protein Recovery in Peak Fraction (%)	Total Protein Recovery (%)
Tris	51	55
Phosphate	67	85

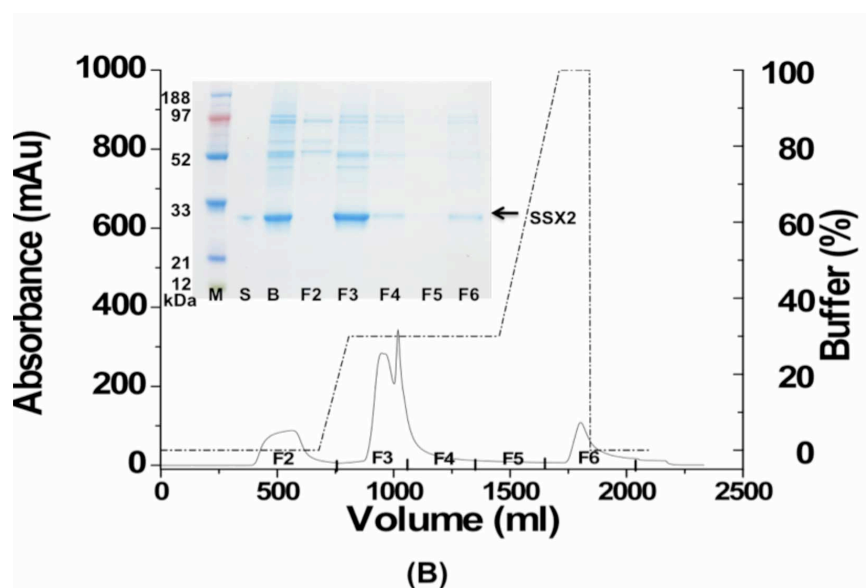
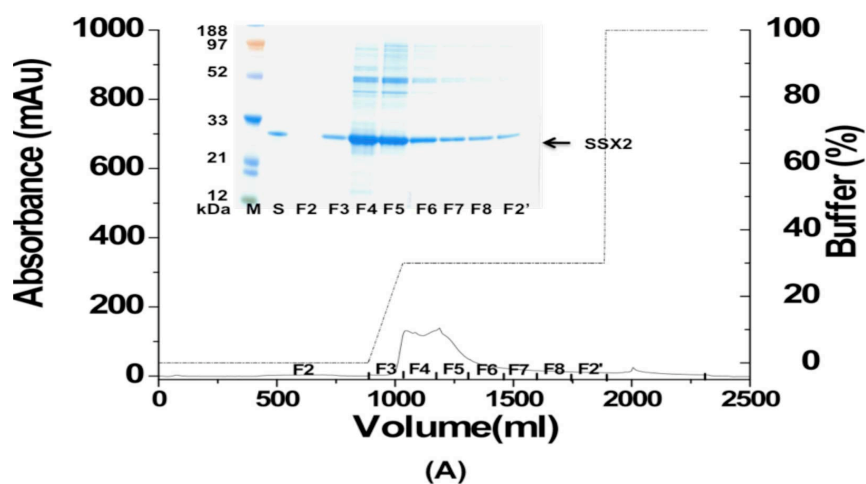


Figure 3-1. Comparison of FPLC loading and elution profiles of SSX2 antigen applied on 150 ml anion exchange column (Q Sepharose HP) by using Tris (A) and Phosphate (B) buffers. Solid line indicates absorbance at 280 nm and dash line represents percentage of buffer that contained 1 M NaCl. Inset: Coomassie-stained SDS-PAGE of fractions from AXC chromatography. M, molecular weight marker; S, SSX2 standard; B, raw material before loading; F2 to F9 indicate different fractions collected during the chromatography.

suggesting that even though protein yield was compromised, the purity of SSX2 was improved in the flow through fraction (Figure 3-2 C). In QHP, however, 91% of SSX2 was eluted in the gradient with an endotoxin concentration of 1250 EU/mg with no significant improvement in protein purity (Figure 3-2 C). QXL was able to remove endotoxin more efficiently than QHP. The average particle size of QXL resin is 90 μm which is about three times larger than QHP (34 μm). In addition, agarose beads of QXL consist of dextran arms conjugated with quaternary amino group. These two characteristics make protein binding and release slower in QXL (16). Our data suggest that the slower binding of SSX2 to the QXL resin may actually reduce the binding between SSX2 and quaternary amino group, eluting SSX2 in the flow through fraction. However, the binding of endotoxin was not affected as indicated by both low endotoxin in the flow through and high concentration of endotoxin in the gradient eluted fraction. This allowed SSX2 to be separated from endotoxin by using QXL resin.

10 L scale development run in cGMP facility

Because the yield was improved by Phosphate buffer and endotoxin removal was enhanced by using a QXL column, a new purification process to produce SSX2 antigen was developed (Figure 3-3). Phosphate buffer was used for chromatographic steps. QXL was added as a final step to polish SSX2 protein including endotoxin removal. A development run using this new process was scaled-up in the Cornell/LICR Biologics cGMP facility. The result of the fermentation was summarized in the Table 3-2 that the OD₆₀₀ of *E. coli* was 166.5 and the wet cell weight reached 280 g/L at the end of the fermentation. A total of 10 L cell broth was harvested for SSX2 purification. The total protein concentration and endotoxin content

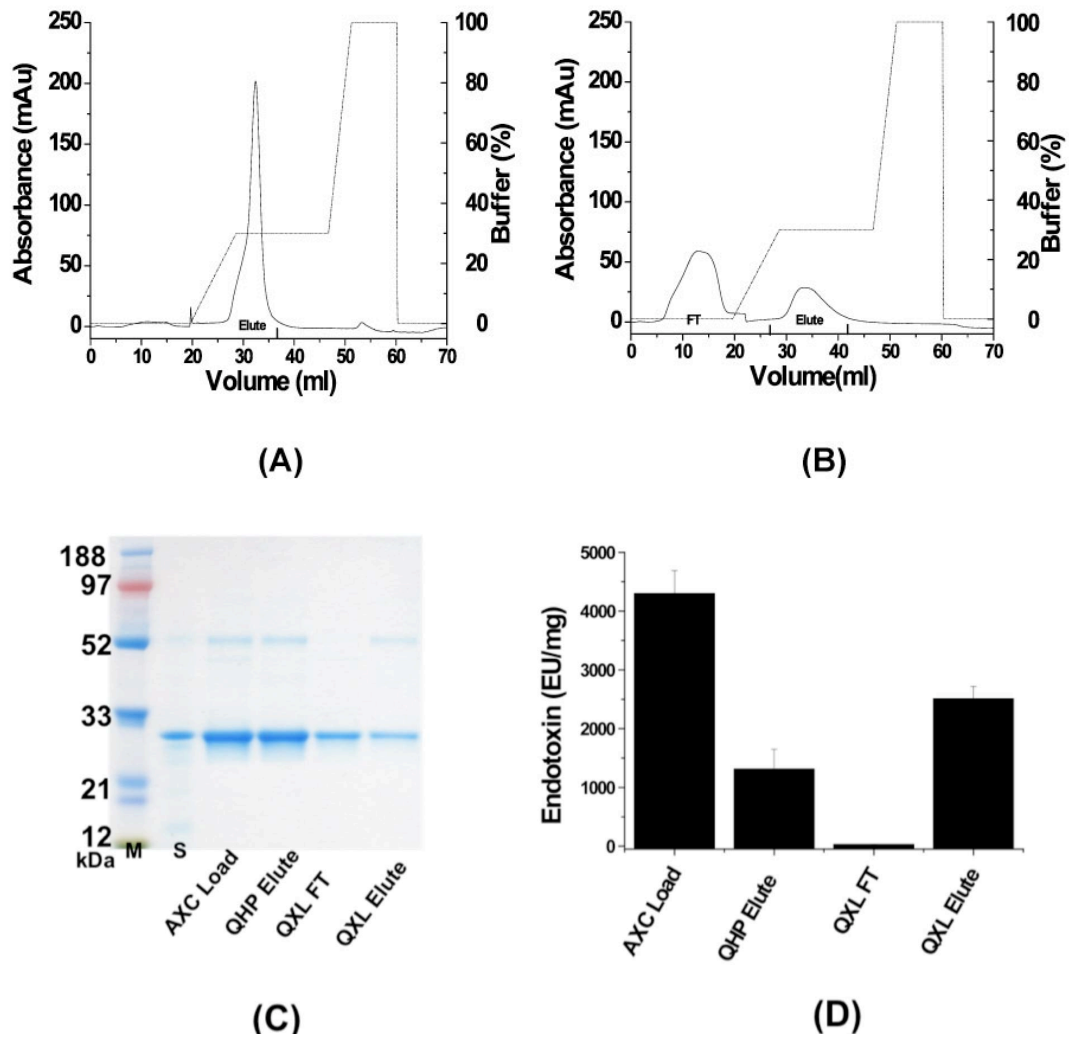


Figure 3-2. Endotoxin removal of SSX2 antigen by different anion exchange resins. After hydrophobic interaction chromatography, SSX2 was loaded on (A) QHP and (B) QXL columns to study the effects of endotoxin reduction (D). SSX2 was mainly eluted in the salt gradient in the QHP column while most of the SSX2 was accumulated in the flow through (FT) in the QXL column. SDS-PAGE (C) also showed the difference in protein purity in these two different anion exchange columns. M, molecular weight standard; S, SSX2 standard.

of each purification step was illustrated in the Table 3-3. The SDS-PAGE and western blot of different purification samples were shown in the Figure 3-4. At the end of purification, a total of 420 mg of SSX2 was produced, and over 97% pure of SSX2 was obtained with endotoxin content of 40 EU/mg. The material produced in this batch was qualified for safety including low endotoxin content, low nickel content and sterility. SSX2 identification was confirmed by MALDI-TOF mass spectroscopy, and its molecular weight was determined to be about 22.4 kDa (data not shown). The results from this development run demonstrated that the new process is plausible to be implemented in our cGMP facility to produce qualified SSX2 for future clinical trials.

Refolding of SSX2

The purified and urea-solubilized SSX2 was refolded on an 5 ml IMAC column. In this process, 5 mg of SSX2 was loaded onto the column and washed with urea buffer. A gradient of 4 M urea and phosphate buffer was applied to the column to gradually changing urea concentration from 4 M to zero in phosphate buffer. The bounded SSX2 was eluted with phosphate buffer containing 500 mM imidazole. The FPLC chromatogram of the refolding process was shown in the Figure 3-5 A. A total of 80% of soluble SSX2 was recovered in the eluted fraction (Table 3-4). Neither sign of cloudiness nor precipitates were found in the eluted fraction (Figure 3-5), indicating that the refolded SSX2 was stable in the phosphate buffer. Since soluble SSX2 had not been characterized in the literature, it was scanned at Far-UV wavelength by a CD spectrometer to analyze its secondary structures (Figure 3-6). The chromatogram was deconvoluted to approximate the fractions of each secondary structure type. The refolded SSX2 was estimated to contain 23% of alpha helix, 19% of beta sheet and 58% random coils compared to 19.5% of alpha helix, 9.3% beta sheet and 72.2%

Table 3-2. Summary of the cell growth and characterizations of SSX2 production strain during the fermentation process.

Test/Procedure	Inoculum	Preinduction	Harvest
OD ₆₀₀	3.89	28.9	166.5
Wet Cell Weight	NA	63.5 g/L	280 g/L
Gram Stain	Rod, Gram Negative	Rod, Gram Negative	Rod, Gram Negative
Microscopic Purity	Rod, No Contamination	Rod, No Contamination	Rod, No Contamination
<i>E. coli</i> Identity	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
Protein	NA	SDS-PAGE & Anti-SSX2	SDS-PAGE & Anti-SSX2
Plasmid DNA	Passed PCR & DNA Sequencing	Passed PCR & DNA Sequencing	Passed PCR & DNA Sequencing

Table 3-3. Summary of total protein concentration and endotoxin content in different purification steps from a 10 L fermentation broth containing SSX2 antigen at CU/LICR cGMP Biologics Facility.

Process	Total Protein (mg)	Endotoxin (EU/mg)
Crude Cell Lysate	217,424	-
IB TRIS Wash Retentate	119,295	-
IB Phosphate Wash Retentate	63,785	-
IMAC Load	90,545	6.0x10 ⁵
IMAC HI	12,155	-
QHP Load	11,614	1.4x10 ⁵
QHP Elute	4,706	7.1x10 ⁴
HIC Load	3,276	-
HIC Elute	840	9.9x10 ³
QXL Load	766	-
QXL FT	420	40

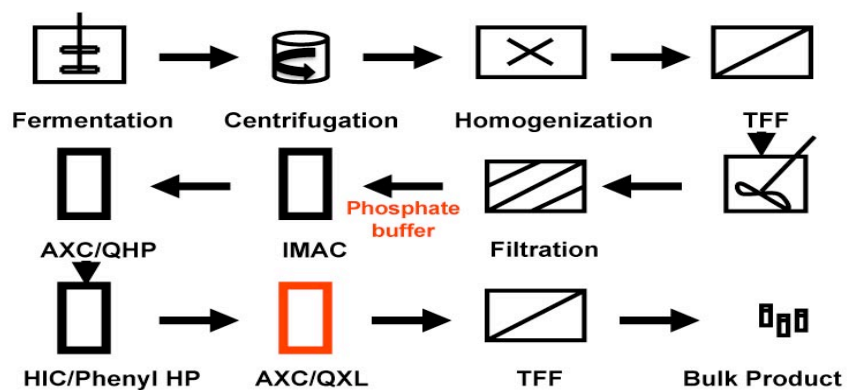


Figure 3-3. A modified SSX2 production process used for a 10L scale development run in Cornell/LICR cGMP Biologics. The red rectangle and wording indicate the modifications made to the previously reported process.

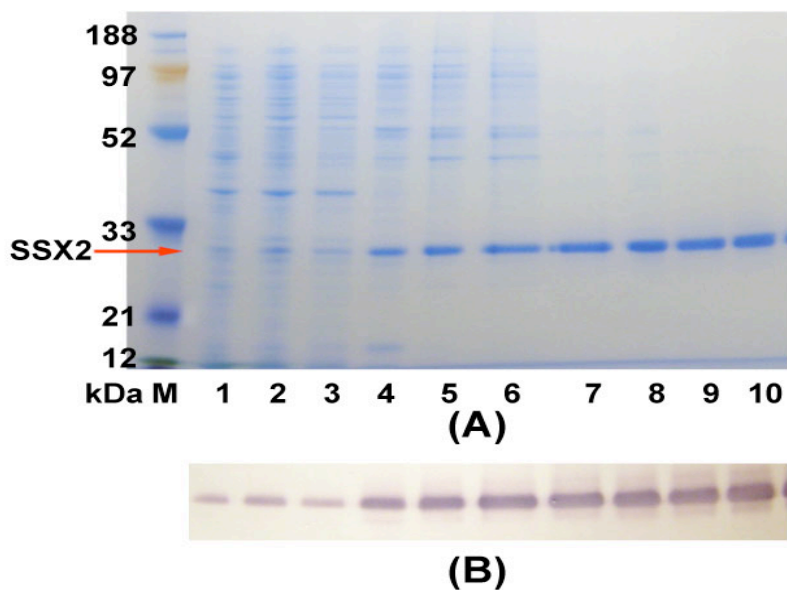


Figure 3-4. SDS-PAGE (A) and Western Blot (B) of SSX2 in different downstream purification steps. Lanes: 1, cell lysate; 2, phosphate wash retentate; 3, IMAC load; 4, QHP load; 5, QHP elute; 6, HIC load; 7, HIC eluate; 8, QXL load; 9, QXL elute; 10, QXL flow through. M, molecular weight marker.

random coils that directly delineate from primary sequence of SSX2 (12).

In order to activate cancer specific cytotoxic T lymphocytes ($CD8^+$) to attack cancer cells, the antigens are delivered to Antigen Presenting Cells such as Dendritic Cells (DC). In DC, the intake antigens are sorted into MHC class I and class II processing pathways that active $CD8^+$ and $CD4^+$ lymphocytes respectively (19). Although $CD8^+$ cells are the main effectors in antitumor immune responses, the activation of $CD4^+$ promotes the growth and help memory maintenance of $CD8^+$ cells (15). Several studies have suggested that addition of intact soluble antigen to DC would majorly in entry of MHC class II processing pathway that activate the $CD4^+$ cells (1, 5). Therefore, the purpose to generate soluble SSX2 is to provide clinical immunologists another format of SSX2 antigen that can be used in combination with urea-solubilized SSX2 to monitor anti-cancer responses both *in vitro* and *in vivo*.

In June 2007, the result of Phase II proof-of-concept clinical trial of CT antigen MAGE-A3 in MAGE-A3 positive patients with stage IB or II Non-Small Cell Lung Cancer was announced. This trial analysis showed a 33% reduction in the relative risk of cancer recurrence following surgery in patients treated with the MAGE-A3, compared to placebo (3). A randomized and controlled Phase III safety and efficacy clinical trail of MAGE-A3 has been carried out. Several Phase I/II clinical trials of another CT antigen NY-ESO-1, to different cancers are also in progress (6). These promising results have shown that active immunotherapy by CT antigens can provide alternative and effective treatment to cancers. Together with more insight and information provided from the clinical trials from these two CT antigens, it is foreseeable that more CT antigens, including SSX2, will be tested for their efficacy as cancer vaccines. In this study, we have improved yield and quality of SSX2, and performed a development run in the Cornell/LICR cGMP Biologics

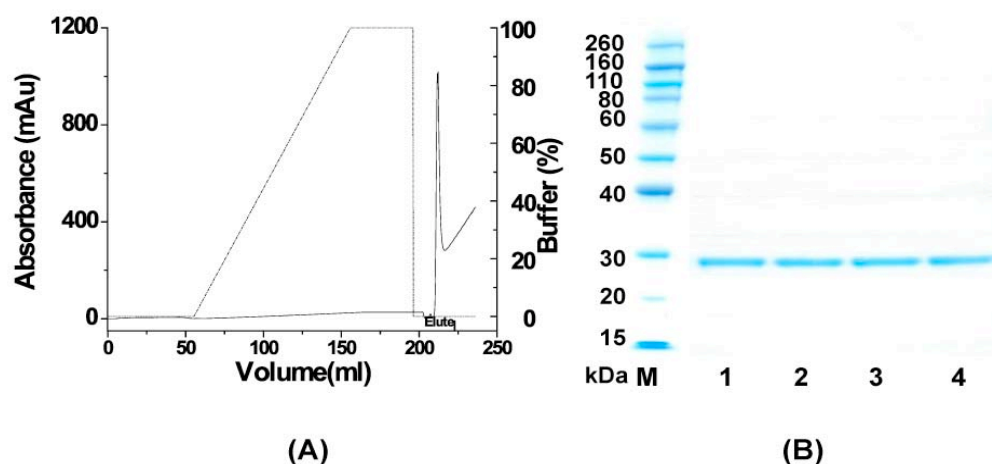


Figure 3-5. Refolding of urea-solubilized SSX2. (A) Refolding of SSX2 antigen by immobilized metal affinity chromatography (IMAC). 10 ml of urea-solubilized SSX2 was loaded on a 5 ml column and protein was refolded by a gradient using 4M urea and 100 mM phosphate buffers. Solid line indicates absorbance at 280 nm and the dash line represents percentage of phosphate buffer, and then eluted with 500 mM imidazole buffer. (B) SDS-PAGE of unfolded SSX2 (1), refolded SSX2 (2) and the supernatant of refolded SSX2 after centrifugation (3), indicating that SSX2 was soluble in 100mM phosphate buffer, and (4) SSX2 standard. M, molecular weight marker.

Table 3-4. SSX2 protein recovery after refolding on the IMAC column

	SSX-2 (mg)	Protein Recovery (%)
IMAC Load	5.7	n/a
IMAC Elute	4.6	80

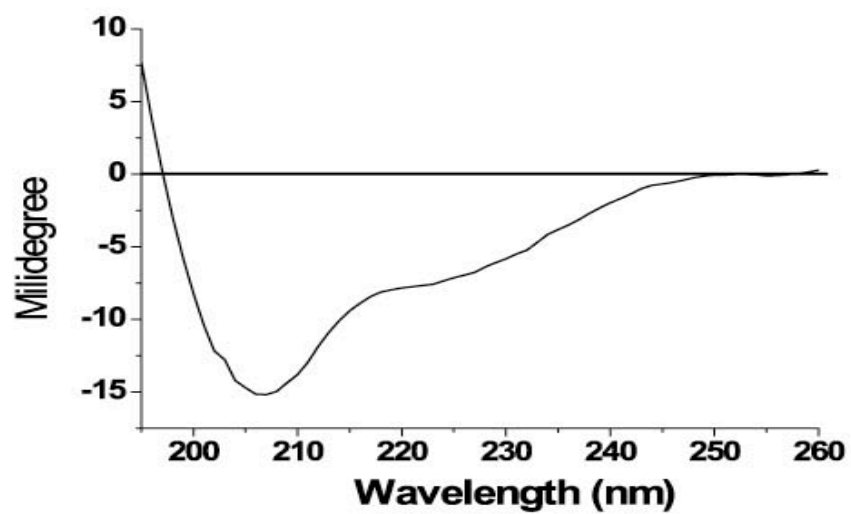


Figure 3-6. Circular Dichroism spectra of refolded SSX2 in 10 mM phosphate buffer pH 7.5. The spectra was deconvoluted to contain 23 % of alpha helix, 19% of beta sheet and 58% of random coils.

facility. These data will provide invaluable information to producing a cGMP grade SSX2 for a proof-of-principle clinical trial in the near future.

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CHAPTER 4

IMPROVED SECRETION OF THE CANCER TESTIS ANTIGEN SSX2 IN *Pichia pastoris* BY DELETION OF ITS NUCLEAR LOCALIZATION SIGNAL

Abstract

The Cancer-Testis (CT) antigen SSX2 was expressed in *Pichia pastoris* as a means to produce a delayed-type hypersensitivity skin test reagent for monitoring SSX2-specific immune response. SSX2 was detected intracellularly in *P. pastoris* despite the addition of the *Saccharomyces cerevisiae* alpha mating factor secretion signal. Increasing SSX2 copy number did not improve its secretion, but did enhance intracellular SSX2 levels. SSX2 with the C-terminal nuclear localization signal (NLS) deleted, (SSX2NORD), however, was secreted. Indirect immunofluorescence indicated that SSX2 with the NLS did not translocate to the nucleus, but accumulated in the endoplasmic reticulum (ER). Experimental results further suggested that SSX2 with the NLS was misfolded in the ER, while deletion of the NLS facilitated correct folding of SSX2 inside the ER and improved its secretion. Production of SSX2NORD was scaled-up to a 2 L fermenter using a fed-batch protocol to maintain methanol at 1 g L⁻¹. Decreasing the cultivation temperature from 25°C to 16°C improved protein stability in the culture supernatant. In this process, after 120 hrs cultivation, the wet cell weight of *P. pastoris* reached 280 mg mL⁻¹ and the yield of SSX2NORD was 21.6 mg L⁻¹.

Huang C-J., Anderson, A. K., Damasceno, M. L., Ritter, G., Old, J. L., Batt A. C. Improved secretion of the cancer testis antigen SSX2 in *Pichia pastoris* by deletion of its nuclear localization signal. Manuscript submitted for publication.

Introduction

The synovial sarcoma X break point 2 or SSX2 gene belongs to a highly homologous family (SSX) that encodes proteins involved in a chromosomal translocation t(X;18)(p11.2;q11.2) characteristically found in 70% of human synovial sarcoma (11). The translocation of synovial sarcoma translocation gene (SYT) on chromosome 18 with an SSX gene (mainly SSX1 and SSX2) creates a SYT-SSX fusion protein which is believed to be a possible transcription activator for aberrant transformation of synovial sarcoma (28). SSX2 is a nuclear protein that contains a bipartite nuclear localization signal (NLS) at its C-terminus that is responsible for the localization of SSX2 and SYT-SSX2 fusion to the nucleus (16). SSX2, also referred to as HOM-MEL-40 or CT5.2a, is an immunogenic tumor antigen identified by the serological analysis of recombinant cDNA expression libraries (SEREX) from a melanoma tumor (38). It is expressed in various cancers while its expression in normal tissue is restricted to the testis. Because of this characteristic expression, SSX2 is also known and classified as a Cancer Testis (CT) antigen (18).

In cancer cells, endogenous cancer antigens are degraded by cytosolic proteasome and the resulted peptides can be delivered to the endoplasmic reticulum (ER) through transporter associated with antigen processing (TAP). In the ER, these cancer-specific peptides are loaded to Major Histocompatibility Complex (MHC) class I molecule and presented on the cancer cell surface by the antigen presenting pathway (32). Since CT antigens are specifically expressed in cancer and testis cells and testis germline cells are MHC class I negative, the expression and activation of CT antigen-specific cytotoxic T lymphocytes is highly tumor specific, making CT antigens ideal targets for cancer immunotherapy. The therapeutic strategy includes delivering CT antigens to Antigen Presenting Cells (APC), for example Dendritic Cells, to induce

and activate CT antigen-specific T lymphocytes to kill the cancer cells presenting CT antigen epitopes (39). Although, SSX2 is a nuclear protein, it has been shown that nuclear proteins are partly transported to cytoplasm for degradation (6), therefore the presentation of SSX2-specific epitopes are found in cancer cells (4, 8). To date, several studies have demonstrated that SSX2 can elicit both humoral and cellular (both CD4⁺ and CD8⁺) mediated immune responses to kill cancer cells (2, 5, 34). For these reasons, there is a considerable interest in producing recombinant SSX2 antigen to clinically evaluate it as a cancer vaccine.

The methylotrophic yeast *Pichia pastoris* has been widely used for production of various recombinant proteins by secretion into the supernatant or by intracellular expression (9). The most commonly used transcriptional regulation for recombinant protein expression is driven by the alcohol oxidase 1 (*AOX1*) promoter, which is strongly induced in medium containing methanol as a sole carbon source. This expression system offers several advantages, such as easy manipulation at the genetic level, high stability of transformants, the capability to culture cells at high cell density, and most importantly, the ability to perform higher eukaryotic protein secretion and post-translational modifications (27). Because *P. pastoris* secretes low levels of endogenous proteins, a secreted heterologous protein constitutes the vast majority of the total protein in the culture supernatant (19). The predominance of the heterologous protein in the supernatant makes following purification steps easier to implement.

However, overexpression of heterologous proteins may saturate the secretory pathway, with proteins accumulating inside the endoplasmic reticulum (ER) where they need to be folded correctly before being transported from the ER to the Golgi apparatus (12). Additionally, the options for secretion of heterologous proteins are normally limited to foreign proteins which are normally secreted by their native hosts

(19). Therefore, one of the challenges in using this expression system is to produce and secrete foreign proteins that are not naturally secreted.

We previously reported the production and purification of SSX2 from *Escherichia coli* C41(DE3) in a 2 L bench-top fermenter with a yield of 236 mg L⁻¹ purified SSX2 (22). In this study, SSX2 produced from *P. pastoris*, rather than *E. coli*, is to be employed for monitoring of SSX2-specific immune responses in a delayed-type hypersensitivity (DTH) skin tests and thereby avoiding measurement of immunological responses to potential bacterial contaminants in the *E. coli*-produced SSX2 vaccine protein (14). We focus on secretion of SSX2 for purification advantages and intend to address the secretion bottlenecks of SSX2 and other nuclear proteins in *P. pastoris*. In this study, we evaluated SSX2 secretion in *P. pastoris* and found that SSX2 is minimally secreted and accumulated inside the cell, mainly in the ER. Deletion of its C-terminal NLS improved SSX2 secretion by facilitating its folding inside the ER. We also report a fermentation protocol to scale-up the secretion of SSX2 without NLS at the yield of 21.6 mg L⁻¹ in the culture media.

Materials and Methods

Strains and plasmids Construction

The SSX2 gene (GenBank Accession No. NM 175698) was obtained from the New York branch of the Ludwig Institute for Cancer Research, cloned into the plasmid pcDNA 3.1 (Invitrogen, Carlsbad, CA). All strains, plasmids and constructs created and used in this study are shown in Table 4-1 and Figure 4-1. *Pichia pastoris* KM71H and plasmid pPICZαA were purchased from Invitrogen. The SSX2 gene was cloned in frame into pPICZαA which uses the *Saccharomyces cerevisiae* alpha mating factor as

a secretion signal and the *AOX1* promoter, resulting in pPICZ α ASSX2. The SSX2NORD construct was amplified by oligonucleotides (5'-GAATTCAACGGAGACGACGCCTTTGCAAGGA-3' and 5'-GCGGCCGCTTATCTCTCGTGAATCTTCTCAGAG-3') using pPICZ α ASSX2 as a template and cloned into plasmid pPICZ α A to create plasmid pPICZ α ASSX2NORD. Both plasmids were transformed into *P. pastoris* KM71H and selected on YPDS plates with different zeocin concentrations to create strains MS03, MS-H and MN-H. The A33 single chain gene was amplified from plasmid pPIC9K-A33scFv (13) and cloned into pPICZ α A to create pPICZ α AA33scFv. The A33scFvNLS construct was amplified by using four oligonucleotides, 5'-CTCGAGAAAAGAGAGGCTGAAGCTGAGCTCCAGA-3', 5'-TTTGGGTCCAGATGAGGAGACGGTGACCAGGGTG-3', 5'-ACCGTCTCCTCATCTGGACCCAAAAGGGGGGAACA-3' and 5'-GGCGGCCGCTTACTCGTCATCTTCCTCAGGGTCG-3', using a PCR touchdown protocol (15). A33scFvNLS was later cloned into pPICZ α A to create pPICZ α AA33scFvNLS. Plasmids of pPICZ α AA33scFv and pPICZ α AA33scFvNLS were transformed into KM71H to generate strains M33 and M33NLS respectively.

Strain cultivation and recombinant protein induction

P. pastoris colonies were transferred to 10 mL YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and grown overnight. The cells were then inoculated in 10 mL YPD medium with an initial optical density (OD₆₀₀) of 0.2 and grew for 24 hrs. Cells were harvested and resuspended in 10 mL BMMY medium (100 mM potassium phosphate, pH 6; 1.34% (w/v) yeast nitrogen base without amino acids, 4 $\times 10^{-5}$ % (w/v) biotin, 1% yeast extract, 2% peptone and 0.5% methanol). Cultures were then grown in a rotary shaker at 30°C /250 rpm for 16 hrs. In one of the

Table 4-1. Strains and plasmids constructed and used in this study

Strain*	Integrated plasmid	Copy number	source
<i>P. pastoris</i> KM71H			Invitrogen
MS03	pPICZαASSX2	1	This study
MS-H	pPICZαASSX2	7	This study
MN-H	pPICZαASSX2NORD	6	This study
M33	pPICZαAA33scFv	1	This study
M33NLS	pPICZαAA33scFvNLS	1	This study

* All strains created in this study were derived from *Pichia pastoris* KM71H

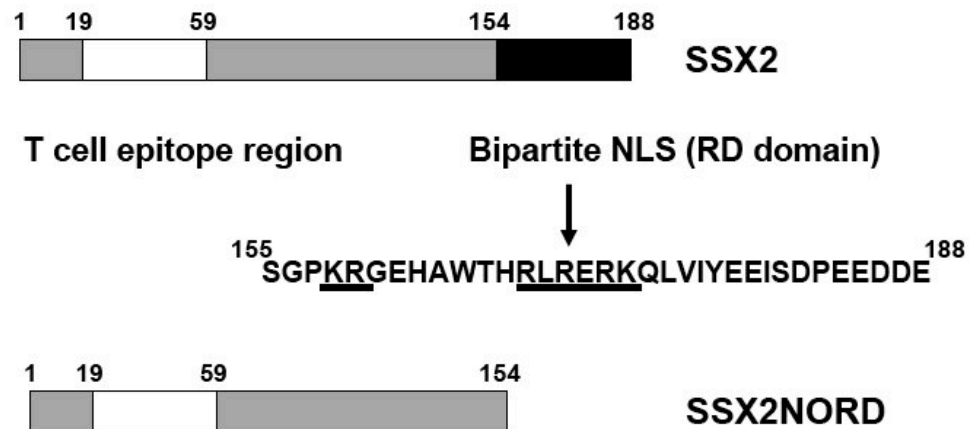


Figure 4-1. Illustration of SSX2 and SSX2NORD constructs. The N-terminal white box covers the MHC class one and two epitopes of SSX2 antigen. The C-terminal black box represents the nuclear localization signal of SSX2 at its C-terminus. The two basic amino acid domains of bipartite NLS are underlined.

particular experiment, cells were harvested, washed with PBS (pH 7.5), resuspended in BMGY medium (100 mM potassium phosphate, pH 6; 1.34% yeast nitrogen base without amino acids, $4 \times 10^{-5}\%$ biotin, 1% yeast extract, 2% peptone and 0.5% glycerol) and cultivated for 12 hrs. Samples were centrifuged at 13,000 x g for 10 min and the culture supernatant and pellet were analyzed.

Southern blot analysis

Transformants, including MS03, MS-H, MN-H, M33 and M33NLS, were grown in YPD medium overnight in a 5 mL culture. Genomic DNA of these transformants was prepared using a Yeaststar genomic DNA kit (Zymo Research, Orange, CA) according to manufacturer's instructions. Different concentrations of plasmids (pPICZ α ASSX2NORD and pPICZ α AA33scFv) corresponding to 1 to 10 copies of SSX2NORD and A33scFv genes were calculated according to the ratio of the size of plasmids (4012 and 4322 bp, respectively) to the genome size of *P. pastoris* (9.6×10^6 bp), and used as standards. (20). Both plasmid and genomic DNA were digested with restriction enzymes, Xho1 and Not1, at 37°C overnight and run on a 0.8% agarose gel. The separated fragments were transferred to a positively charged nylon membrane (Pall, East Hills, NY) overnight. DIG-labeled probes for both SSX2NORD and A33scFv genes were generated using a PCR DIG Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN). After DNA transfer, the blot was later rinsed, UV-immobilized and hybridized with DIG-labeled probe at 48°C for 6 hrs. After washing, the blot was detected with anti-DIG-Fab alkaline phosphatase conjugate and developed using a DIG nucleic acid detection kit (Roche Applied Science) according to manufacturer's instructions. DNA fragments on the developed membrane were then scanned, selected and plotted by Image J densitometry software (<http://rsb.info.nih.gov/ij/>) and the copy number was determined by the constructed

standard curve.

Indirect immunofluorescence and confocal microscopy

Induced *P. pastoris* cells were fixed in 5 mL 100 mM potassium phosphate buffer (pH 6.5) containing 5% formaldehyde and incubated at room temperature for 1.5 hrs. Cells were washed twice with solution B (1.2 M sorbitol, 100 mM potassium phosphate, pH 7.4) and converted to spheroplasts by adding 20 μ L zymolyase (1 mg mL⁻¹) in 500 μ L solution B. Washed cells were placed on L-polylysine coated slides and permeabilized in -20°C MeOH for 6 min followed by -20°C acetone for 30 sec and blocked in PBS/0.1% BSA for 20 min. The cells were then incubated with 5 μ g mL⁻¹ mouse anti-SSX2 antibody and 10 μ g mL⁻¹ rabbit anti-Kar2p antibody. After 1.5 hrs incubation at room temperature, cells were washed three times with PBS/0.1% BSA and incubated with 20 μ g mL⁻¹ of Alexa546-conjugated goat anti-mouse IgG for 1.5 hrs for indirect immunofluorescence. For confocal microscopy, slides were incubated with 20 μ g mL⁻¹ of Alexa488-conjugated goat anti-mouse IgG and 20 μ g mL⁻¹ of Alexa546-conjugated goat anti-rabbit IgG for 1.5 hrs. Cells were washed three times and images were examined. For nucleus staining in immunofluorescence, 10 μ g mL⁻¹ of Hoechst dye was added into the cells and incubated for 30 min during the secondary antibody incubation. Immunofluorescence microscopy was performed using an Olympus BX61 epifluorescent microscope (Olympus America, Center Valley, PA). Images were acquired using a Cooke SensiCam and Slidebook software (Intelligent Imaging, Santa Monica, CA). Confocal microscopy was performed using a Leica TCS SP2 system (Leica Microsystems, Bannockburn, IL) and processed using Leica Lite software. Images of different emission wavelengths were overlaid to examine co-localization.

Protein sample preparations from *P. pastoris*

A 1 mL aliquot of cells from induced strains (Table 4-1) was centrifuged at 13,000 x g for 10 min. The culture supernatants were collected and proteins were precipitated by adding 10 % (v/v) trichloroacetic acid (TCA) and incubated on ice for 30 min. Supernatant was centrifuged, carefully removed and washed with 200 μ L cold acetone. Acetone was then removed by centrifugation and protein pellets were dried at room temperature for 30 min. A 100 μ L aliquot of 1X LDS sample buffer (Invitrogen) was added to solubilize the protein pellets and the resulted protein sample was loaded on the gel. Cytoplasmic (soluble) and membrane-associated (insoluble) fractions were collected as previously described (12). Briefly, the previous cell pellets were washed in the phosphate-buffered saline (PBS) at pH 7.4 and resuspended in 100 μ L of yeast breaking buffer (50 mM sodium phosphate, pH 7.4; 1 mM phenylmethyl sulfonyl fluoride; 1 mM EDTA and 5% (v/v) glycerol). 100 μ L of acid-washed glass beads were added and vortexed at maximum speed seven times for 1 min with 1 min intervals on ice to disrupt the cells. The lysate was centrifuged at 14,000 x g for 30 min at 4°C and the supernatant was collected as the cytoplasmic protein fraction. The remaining pellet was resuspended in yeast breaking buffer containing 2% sodium dodecyl sulfate (SDS) and centrifuged at 4,000 x g for 5 min. The supernatant was collected as the membrane-associated fraction. For whole cell fraction preparation, 100 μ L of lysis buffer (50 mM sodium phosphate, pH 7.4; 1 mM phenylmethyl sulfonyl fluoride; 1 mM EDTA and 5% (v/v) glycerol; 0.1% Triton X100) and 100 μ L of acid-washed glass beads were added and vortexed. The cells were then centrifuged and the supernatant was collected as the whole cell fraction (30).

SDS-PAGE and immunoblotting

TCA precipitated culture supernatants, cytoplasmic, membrane-associated and

whole cell fractions were normalized for same cell optical density before analysis. Samples were analyzed by electrophoresis on 12% SDS-polyacrylamide gels (Invitrogen). After electrophoresis, the gel was stained by SimplyBlue SafeStain (Invitrogen). For western blot, the proteins were electroblotted on to a nitrocellulose membrane and SSX2 or SSX2NORD was detected with 2.5 µg of murine anti-human SSX2 monoclonal antibody using a WesternBreeze kit (Invitrogen). Kar2P (BiP) detection was carried out by adding a 1:2,000 dilution of rabbit anti-Kar2p antibody (given by Dr. Barlowe). All assays were performed according to manufacturer's instructions. Protein concentrations and protein level comparisons were estimated from scanned images of the developed western blots with Image J densitometry software (<http://rsb.info.nih.gov/ij/>). Western blots were scanned and images of the protein bands were selected, plotted and compared with standards or a desired band.

Fermentation

A 1 mL of overnight culture of strain MN-H was used to inoculate 100 mL of BMG medium (100 mM potassium phosphate, pH 6, 1.34% (w/v) YNB, 4×10^{-5} % (w/v) biotin and 1% glycerol) and grown at 30°C for 16 hrs until the OD₆₀₀ reached 10. This culture was used to inoculate the fermentor (Bioflo 3000, New Brunswick Scientific, Edison, NJ). The fermentation was carried out in a 2 L bench-top fermentor with an initial volume of 1 L modified BMGY medium with 5% (v/v) glycerol and supplemented with 4.35 mL PTM1 trace salts (24 mM CuSO₄, 0.53 mM NaI, 19.87 mM MnSO₄, 0.83 mM Na₂MoO₄, 0.32 mM boric acid, 2.1 mM CoCl₂, 0.15 mM ZnCl₂, 0.23 M FeSO₄ and 0.82 mM biotin). AFS-Biocommand Bioprocessing software version 2.6 (New Brunswick Scientific) was used for data acquisition and parameter control. During the fermentation, dissolved oxygen was maintained at 40% air saturation and was controlled by a D.O. cascade of agitation followed by pure

oxygen sparging when needed. At the end of the batch phase, indicated by a D.O. spike, the culture was induced with a methanol feed (100% methanol with 12 mL PTM1 salts/L) and the methanol concentration was controlled at 1 g L⁻¹ throughout the fermentation. A methanol sensor, sensor unit and PID control was used to monitor and control methanol concentration (13). Samples were taken for analysis of SSX2NORD and wet cell weight (WCW) during the fermentation. For WCW, a 1 mL of the broth was collected and centrifuged at 13,000 x g for 10 min. The supernatant was collected for SSX2NORD analysis, and the cell pellet was weighed to determine WCW.

Results

SSX2 accumulates intracellularly despite the addition of signal peptide

In order to express and secrete SSX2 into the culture media, the SSX2 gene was cloned into pPICZ α A plasmid which carries the *S. cerevisiae* alpha mating factor as a secretion signal. The plasmid, pPICZ α ASSX2, was transformed into competent *P. pastoris* KM71H cells and the transformants were selected on YPDS plates containing 100 μ g/ml of zeocin. Several transformants including MS03, with one copy of pPICZ α A SSX2 integrated, were cultivated, induced and fractionated into three different cellular fractions. Immunoblot analysis of different fractions revealed that SSX2 was poorly expressed, accumulated in the intracellular membrane-associated fraction and that the alpha mating factor signal peptide was not processed. No SSX2 was found in the culture supernatant (Figure 4-2). A high copy number strain MS-H, with seven copies of pPICZ α A SSX2 integrated, was then selected to test whether increasing expression of SSX2 would facilitate its secretion. After induction for 16 hrs, the expression level of SSX2 found in intracellular membrane-associated fraction

increased approximately 49-fold when compared with strain MS03. SSX2 was degraded in the soluble cytoplasmic fraction. However, there was only a minute amount of degraded SSX2 detected in the culture supernatant fraction (Figure 4-2). Thus, we concluded that SSX2 was barely secreted by *P. pastoris*.

Deletion of nuclear localization signal (NLS) improves SSX2 secretion

Because SSX2 is a nuclear protein, it was hypothesized that SSX2 was translocated to the nucleus, thus inhibiting its secretion. To confirm this hypothesis, a construct of SSX2 without the NLS was created (SSX2NORD) and the plasmid pPICZ α ASSX2NORD was transformed into competent *P. pastoris* KM71H cells. A high copy number strain MN-H, with six copies of pPICZ α A SSX2NORD, was selected and SSX2NORD expression and secretion levels were compared with the native SSX2 expression strain, MS-H. As shown in the Figure 4-3, the SSX2NORD in the membrane-associated fraction decreased by 80% as compared to the SSX2 found in MS-H strain. There was no sign of SSX2NORD degradation in the soluble cytoplasmic fraction. Importantly, SSX2NORD was found in the culture supernatant at a concentration of 2.1 mg L⁻¹ as compared with strain MS-H strain that only had a minute amount of degraded SSX2 in its culture supernatant. Therefore, there was less SSX2 accumulated intracellularly and the secretion of SSX2 was significantly improved when the NLS region was deleted.

SSX2 is not targeted to the nucleus, but accumulates within the ER

Since there are two competing signal peptides, the N-terminal alpha mating factor secretion signal and the C-terminal NLS on SSX2, indirect immunofluorescence was performed to localize it and to determine if its translocation to the nucleus was inhibiting secretion. Unexpectedly, as shown in Figure 4-4, most of the SSX2 did not

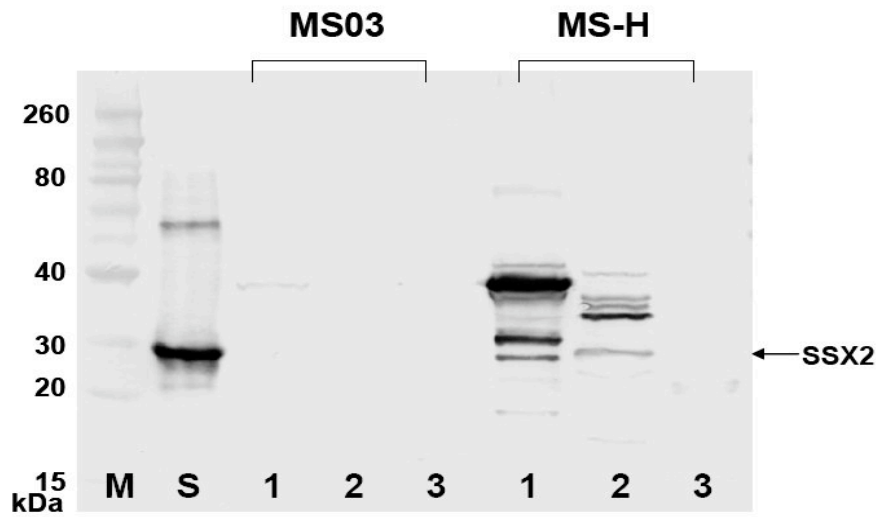


Figure 4-2. Secretion of SSX2 at different copy numbers. Western blot of different cell fractions of MS03 and MS-H strains that have been induced for SSX2 secretion in BMMY medium for 16 hrs. M, molecular weight marker; S, SSX2 standard; 1, membrane-associated fraction; 2, cytoplasmic fraction; 3, culture supernatant fraction.

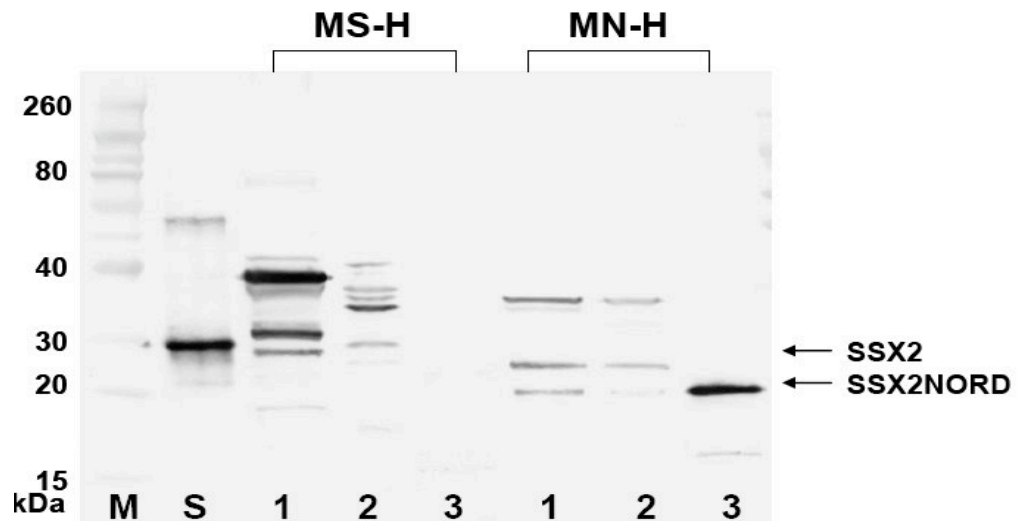


Figure 4-3. Effect of deletion of C-terminal NLS on SSX2 secretion. Western blot of different cell fractions of MS-H and MN-H strains that have been induced for SSX2 and SSX2NORD secretion, respectively, in BMMY medium for 16 hrs. M, molecular

co-localize with nucleus, indicating that the C-terminal NLS is not functioning to direct it to the nucleus. Additionally, as shown in the Figure 4-4B, SSX2 accumulated inside the cell, suggesting that SSX2 is possibly retained inside the ER. Confocal microscopy was performed to co-localize intracellular SSX2 with the ER-resident protein Kar2p. SSX2 did co-localize with Kar2p and mainly accumulated inside the ER (Figure 4-5). Thus, SSX2 was targeted to ER by its N-terminal secretion signal. For MN-H strain, some SSX2NORD also co-localized with Kar2p, but it was not mainly accumulated in the ER and was instead visualized as small dots throughout the cell (Figure 4-6). Some of the SSX2NORD might be in the Golgi complex or in the secretory vesicles that are in the process of secretion.

Unfolded protein response is induced and intracellular accumulated SSX2 is irreversible for secretion

It is known that accumulation of unfolded and misfolded proteins in the ER induces an unfolded protein response (UPR), triggering up-regulation of various ER-resident proteins, including Kar2p, calnexin and protein disulfide isomerase (PDI) (37). Cultures of MS-H, MN-H, KM71 H strain, and KM71H with addition of 10 mM Dithiothreitol (DTT) were induced in BMMY medium for 16 hrs and intracellular Kar2p levels were compared. DTT is an ER stressor that inhibits correct disulfide bond formation, thereby inducing the UPR in cells (33) and was used as a positive control. After induction, Kar2p levels in MS-H and MN-H strains were 3.5 and 2.3 fold higher than the negative control (KM71H) respectively, indicating that the UPR was initiated in both strains (Figure 4-7). Nevertheless, UPR was more intensively induced in the MS-H strain. The Kar2p level in MS-H strain was similar to the positive control which its Kar2p level was 3.9 fold higher than the negative control. T

The possibility of whether the intracellular accumulated SSX2 and

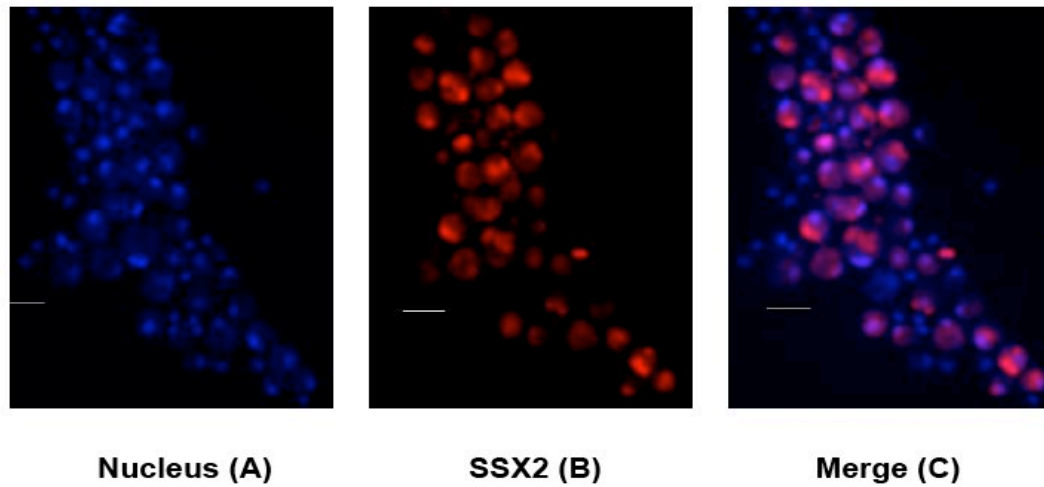


Figure 4-4. Immunofluorescence analysis of the location of SSX2 inside the strain MS-H. After cells were induced for 16 hrs. Samples were labeled with Hoechst dye (A) for nucleus and Alexa fluor 546 conjugated with goat anti-mouse antibody (B) for SSX2. Images were overlapped in (C). Scale bar, 5 μ m

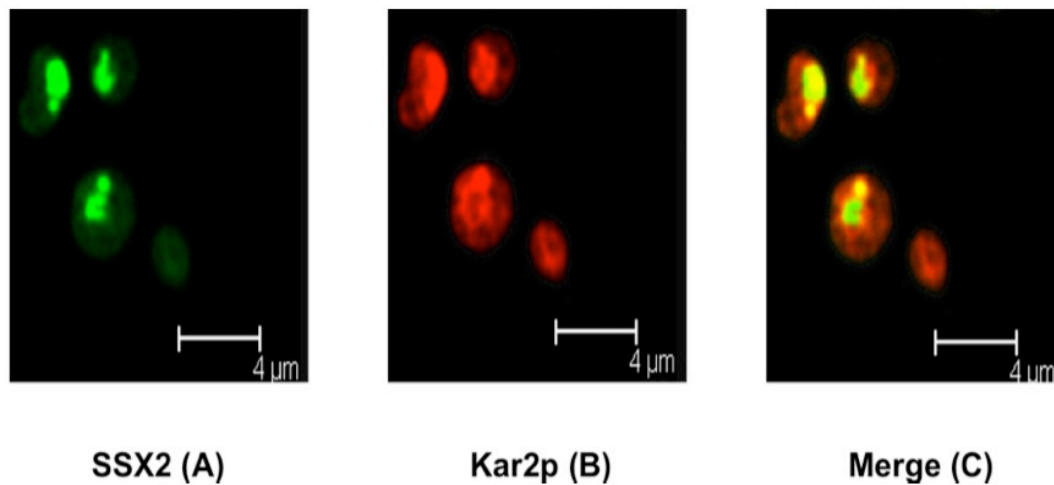


Figure 4-5. Confocal microscopy analysis of the co-localization of SSX2 and ER-luminal protein Kar2p or BiP in strain MS-H. SSX2 was stained with Alexa fluor 488 conjugated with goat anti-mouse antibody (A) and Kar2p was stained with Alexa fluor 546 conjugated with goat anti-rabbit antibody (B). Images were overlapped in (C).

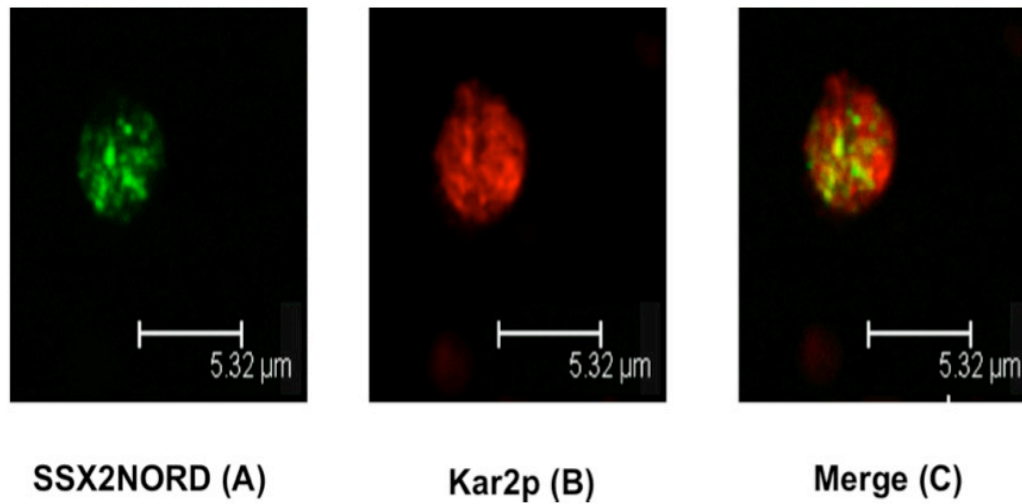


Figure 4-6. Confocal microscopy analysis of the co-localization of SSX2NORD and ER-luminal protein Kar2p or BiP in strain MN-H. SSX2NORD was stained with Alexa flour 488 conjugated with goat anti-mouse antibody (A) and Kar2p was stained with Alexa flour 546 conjugated with goat anti-rabbit antibody (B). Images were overlapped in (C).

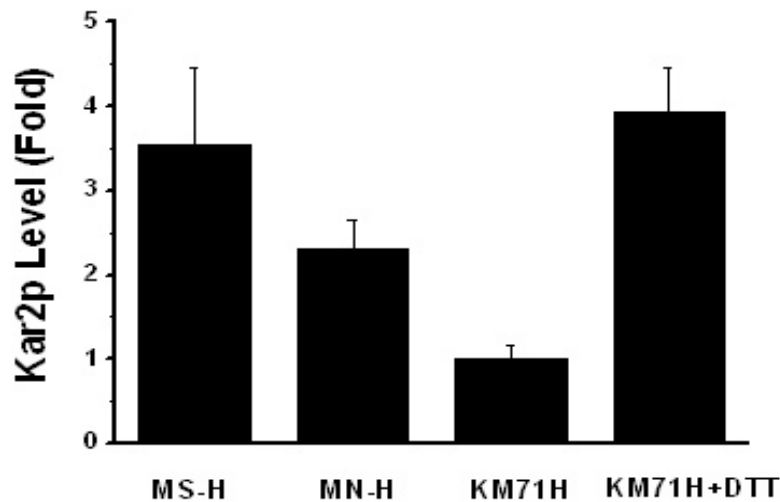


Figure 4-7. Intracellular membrane-associated Kar2p (BiP) levels in MS-H, MN-H and KM71H strains after methanol induction for 16 hrs. Cells were normalized before loading on immunoblots. Image analysis was performed using immunoblots from two independent experiments. Kar2p level of KM71H strain was normalized as one fold and compared its level with other strains and addition of 10 mM DTT that used as a positive control for measuring UPR response.

SSX2NORD could be processed by cells for secretion was tested. After induction for 16 hrs, cells of MS-H and MN-H were centrifuged, washed to remove residual methanol and resuspended in fresh BMGY medium to stop SSX2 expression. MS-H and MN-H were further cultivated in BMGY for additional 12 hrs, and whole cell and culture supernatant fractions were analyzed for SSX2 and SSX2NORD in two strains at different time points. The intracellular SSX2 in MS-H was degraded over time as its intracellular levels decreased and not secreted (Figure 4-8A). In the same experiment, however, intracellular SSX2NORD in the MN-H strain was not degraded because the increased presence of SSX2NORD was found in the culture supernatant over time (Figure 4-8B). Together with previous results, the intracellular SSX2 in the ER could not be secreted and therefore degraded. On the contrary, in MN-H strain, intracellular SSX2NORD could be processed for secretion.

Fusion of the SSX2 NLS inhibits the secretion of protein in *P. pastoris*

To further characterize the role of NLS in recombinant secretion in *P. pastoris*, a single chain antibody, A33scFv, which is normally secreted was tagged with SSX2 NLS at its C-terminus (A33scFvNLS). Its secretion was compared with the native A33scFv after induction in BMMY medium for 16 hrs. As indicated in Figure 4-9, the secretion of A33scFv decreased by 70% when tagged with NLS. This study suggested that the NLS of SSX2 may decrease recombinant protein secretion in *P. pastoris*.

Scale-up production of SSX2 without NLS

SSX2NORD production in *P. pastoris*, was scaled to a 2 L fermentor. However, when MN-H strain was cultivated at 25°C, pH 6 and induced for 48 hrs, less than 1.3 mg mL⁻¹ of SSX2NORD was found in the culture supernatant and SSX2NORD was detected intracellularly (Figure 4-10A). It was possible that secreted

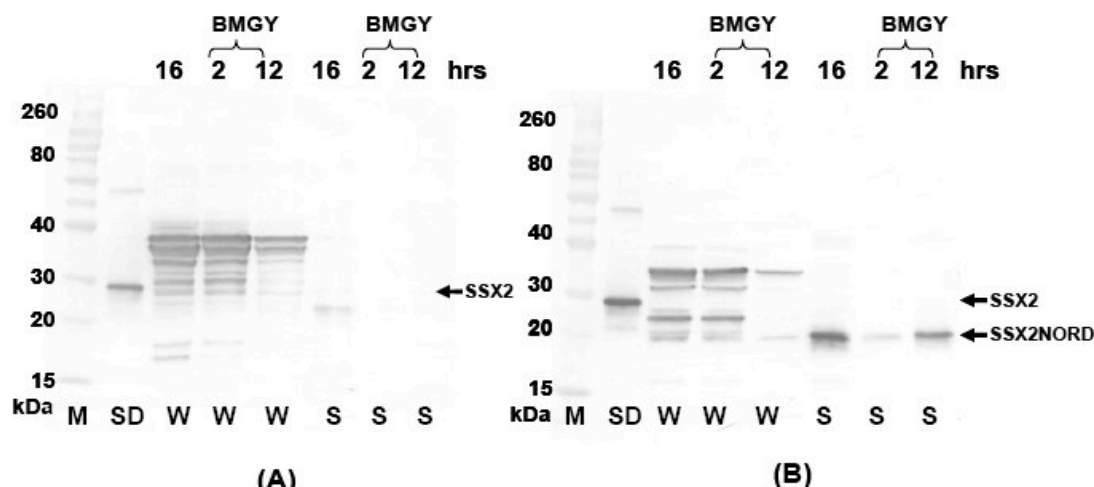


Figure 4-8. The different fates of intracellular SSX2 and SSX2NORD over time after their expressions were stopped. Both MS-H (A) and MN-H (B) were induced in BMMY medium for 16 hrs. After induction, cells were later centrifuged, washed and continued to cultivate in BMGY medium to stop protein expression and cultivated for another 12 hrs. Samples were taken at different time points to analyze intracellular and culture supernatant SSX2 and SSX2NORD respectively. M, molecular weight marker; SD, SSX2 standard; W, whole cell fraction; S, culture supernatant fraction.

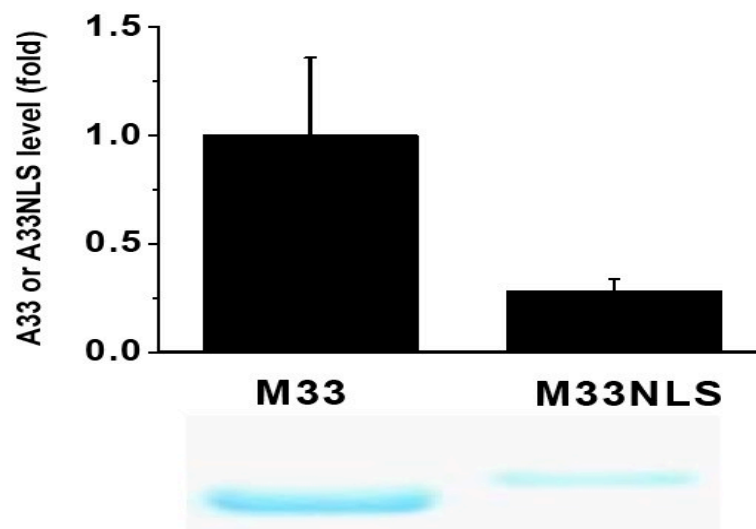


Figure 4-9. Tagging NLS to A33scFv reduced its secretion. After induction for 16 hrs in BMMY medium, supernatant of M33 and M33NLS were collected, analyzed and compared on the coomassie-stained SDS-PAGE. A33scFv level of M33 strain was normalized as one fold and compared its level with A33scFvNLS of M33NLS strain.

SSX2NORD was not stable in the culture supernatant and was susceptible to degradation under the aforementioned conditions. To improve SSX2NORD stability in the culture supernatant, the cultivation temperature was changed from 25°C to 16°C to decrease the protease activity in the culture media (24). The corresponding SDS-PAGE and immunoblot of the samples from these fermentation conditions is shown in Figure 4-10B. The stability of SSX2NORD in the culture supernatant was improved significantly by lowering the cultivation temperature. The production yields of SSX2NORD in the culture supernatant at 72, 102 and 120 hrs were 3.5, 28.6 and 21.6 mg L⁻¹, respectively. The fermentation profile of SSX2NORD at temperature 16°C, pH 6.0 was shown in Figure 4-11. The batch phase was continued for 48 hours until the glycerol was consumed, which was indicated by a sharp rise in the dissolved oxygen and a decrease in agitation. The methanol feed then started at a steady-state concentration of 1 g L⁻¹ until the end of the fermentation. In this process, after 120 hrs cultivation, the wet cell weight of strain MN-H reached 280 mg mL⁻¹ and the SSX2NORD was produced at a yield of 21.6 mg L⁻¹ in the culture media.

Discussion

P. pastoris is a robust system to express and secrete recombinant proteins. However, many studies including our unpublished data have suggested that some proteins are poorly secreted by *P. pastoris* (31, 40). In this study, when SSX2, a nuclear protein and cancer antigen, was expressed and secreted in *P. pastoris*, the protein accumulated intracellularly even with the addition of the alpha mating factor secretion signal. Several approaches have been explored in our lab to improve SSX2 secretion including changes in cultivation conditions (e.g., different cultivation temperature, media and pH) and different secretion signals. However, none of these

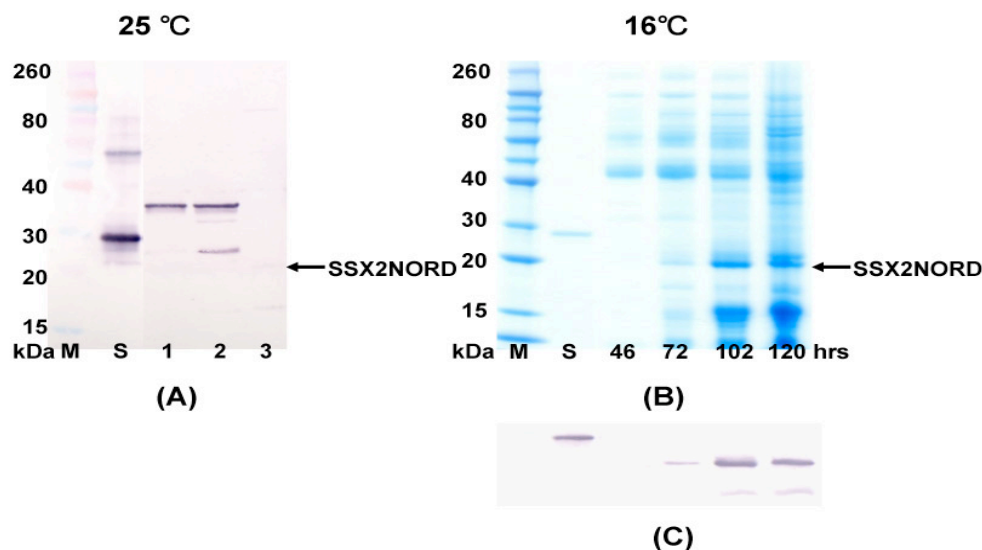


Figure 4-10. Effect of different cultivation temperatures on secretion of SSX2NORD in strain MN-H. (A) Western blot of different cell fractions of MN-H cultivated at 25°C after induction for 48 hrs. (B) Coomassie-stained SDS-PAGE and (C) western blot of supernatant of strain MN-H cultivated at 16°C. M, molecular weight marker; S, SSX2 standard; 1, membrane associated fraction; 2, cytoplasmic fraction; 3, supernatant. In (B) and (C), lane 3 to 6 indicate samples taken at different time points.

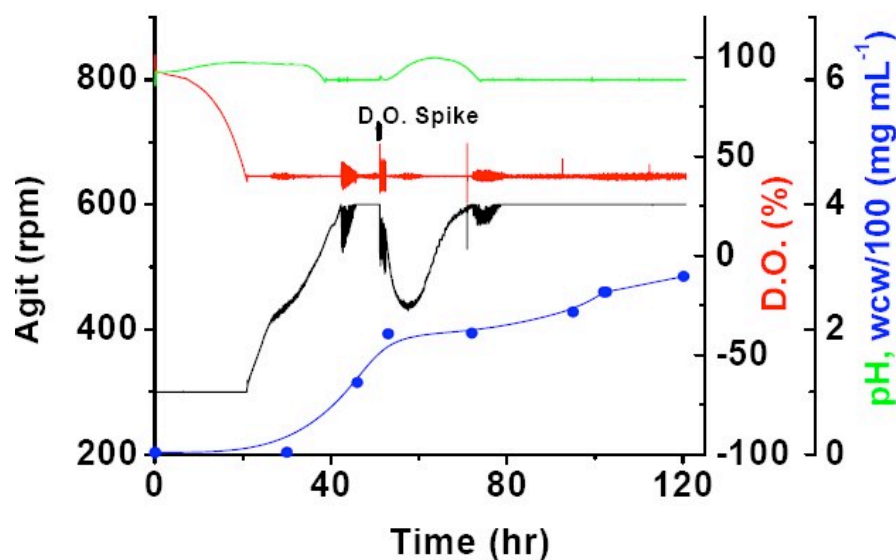


Figure 4-11. Fermentation profile of strain MN-H cultivated at 16°C using a 2 L fermentor. During the fermentation, dissolved oxygen (D.O.) was maintained over 40% of air saturation and pH was maintained around 6. After induction, methanol concentration was maintained at 1 g L⁻¹.

approaches improved SSX2 secretion (data not shown), indicating that the apparent poor secretion is inherent to the protein itself. In addition, increased copy number only led to more SSX2 accumulation inside the cells, thus showing that SSX2 is also one of the recombinant proteins that are poorly secreted by *P. pastoris* despite the addition of a secretion signal.

dos Santos and collaborators have demonstrated that SSX2 has a bipartite NLS at its C-terminus which is also responsible for its and SYT-SSX2 fusion protein's translocation from the cytoplasm to the nucleus (16). A study that investigated secretion of human tumor suppressor p53 in *P. pastoris* has hypothesized that NLS at the C-terminus of p53 might cause its intracellular accumulation (1). It is reasonable to speculate that the C-terminal NLS of SSX2 may inhibit its secretion by translocation of SSX2 to the nucleus. SSX2 without NLS (SSX2NORD) was constructed, expressed and secreted in *P. pastoris* and deletion of the NLS significantly improved SSX2 secretion from no secretion to 2.1 mg L⁻¹ (Figure 4-3). Immunofluorescence however showed that intracellular SSX2 did not localize in the nucleus, suggesting that the NLS does not function to direct it there. In fact, most of the SSX2 was translocated to and accumulated intracellularly mainly in the ER (Figure 4-5). The mechanism of improved secretion of SSX2NORD was further investigated.

Overexpression of heterologous proteins in eukaryotic cells can cause saturation of the secretory pathway, mostly accumulating proteins inside the ER (36). The accumulation of misfolded proteins inside the ER or an increase in protein traffic through the ER will induce UPR to help the cells to cope with the ER stress by up-regulating levels of ER chaperones (e.g., BiP, calreticulin, calnexin), protein-folding enzymes (e.g., PDI, FKB2) (10). Therefore, an increase in Kar2P level is a marker for UPR activation in yeast and mammalian cells (26). We found that intracellular Kar2p

levels in MS-H was 3.5 fold higher than the KM71H strain and indicates that UPR was initiated to help cells cope with secretion stress. In order to know the fate of intracellular accumulated SSX2, methanol was removed and fresh BMGY medium containing glycerol was added. It is known that the *AOX1* promoter is induced by methanol and repressed by glycerol (19, 23), thus, recombinant protein expression is stopped by changing the media to BMGY. The study suggests that the intracellular SSX2 cannot be secreted after protein expression was stopped. Instead, it was degraded over time inside the cells presumably by ER associated degradation (ERAD) which dislocates misfolded protein across the ER membrane for degradation by cytosolic proteasomes (35). Together, these results suggested while expressing SSX2, it was misfolded and accumulated in the ER, alarming cell to initiate UPR and ERAD responses.

On the other hand, while compared to SSX2, expression and secretion of SSX2NORD in MN-H strain displayed different cellular responses. Deletion of NLS improved SSX2 secretion, and decreased intracellular SSX2NORD. UPR was also induced to a lesser extent in SSX2NORD expressing cells, 2.3 fold higher than the control strain, which is possibly caused by intracellularly accumulated SSX2NORD (Figure 4-3). These results indicated that deletion of NLS improved SSX2 folding in the ER, thus facilitated its secretion. Nevertheless, when protein expression was stopped, the intracellular SSX2NORD was secreted by the cells over time, indicating that by giving cells more time, it will be able to fold and secrete intracellularly accumulated SSX2NORD. These experiments also show that even though the secretion was improved in SSX2NORD, ER folding or post-ER translocation could still be a limiting step for SSX2NORD secretion. This is also supported by the confocal microscopy which showed that some of the SSX2NORD accumulated in

cells might be in the vesicles. These vesicles might be secretory vesicles waiting for secretion. However, it is also possible that some vesicles were targeted to vacuole for degradation as seen in other recombinant protein secretion (21), which explains low level secretion of SSX2NORD.

To further characterize the role of NLS in recombinant protein secretion in *P. pastoris*, the NLS of SSX2 was tagged to a well-secreted protein, single chain antibody A33scFv, which has been demonstrated to reach a level of 4 g L⁻¹ in fermentor cultures (13). The fusion of the SSX2 NLS reduced the secretion of A33scFv by 70% as compared to the native A33scFv. The mRNA expression levels of A33scFv and A33scFvNLS in M33 and M33NLS strains did not have appreciable difference (data not shown). We conclude that NLS plays a negative role in recombinant protein secretion in *P. pastoris*. It is known that the NLS of nuclear proteins are exposed on the protein surface in order to interact and be recognized by importin alpha and importin beta for successful nuclear transportation (7, 17). In our case, it is possible that exposed NLS on the surface inhibits SSX2 and A33scFvNLS folding in the ER, thus preventing or decreasing their secretion, respectively.

SSX2NORD produced in this study can be used as a DTH skin test reagent as well as another format of SSX2 cancer vaccine. Several studies have identified that the important epitopes of SSX2 are located at the N-terminus of the protein. The region of 41-49 amino acid residues of the SSX2 protein is recognized by the tumor reactive CD8⁺ T lymphocytes in association with MHC class I allele HLA-A2. The region of 19-59 amino acid residues of SSX2 was later identified to be recognized by specific CD4⁺ T lymphocytes in association with MHC class II alleles HLA-DR3, 4 and 11 and DPI (2, 3, 5, 29). Because the NLS region is downstream of the two important epitopes described above, its removal should not impact its utility as another format of

SSX2 cancer vaccine.

Since there are two potential applications of SSX2NORD, the production of SSX2NORD was scaled-up in a 2 L bench top fermentor. Surprisingly, there was only a minute amount of SSX2NORD found in the culture supernatant when cells were cultivated at 25°C, pH 6, while intracellular SSX2NORD was observed at all times. It is well-known that high cell density fermentation of *P. pastoris* is accompanied with significant cell lysis, releasing intracellular proteins into the culture medium, including vacuolar proteases that cause secreted recombinant protein degradation (24). It is possible that secreted SSX2NORD was degraded during the fermentation process. To minimize proteolytic instability of secreted SSX2NORD, the MN-H strain was cultivated at 16°C to decrease protease activity (25). By changing the cultivation temperature from 25°C to 16°C, the stability of SSX2NORD in the supernatant was improved significantly; however signs of degradation were still observed as SSX2 degradation products were shown in the western blot (Figure 4-10C). This indicates that decreasing the culture temperature can improve SSX2NORD stability, which likely due to decreasing protease activity in the culture supernatant. In this process, it was estimated that a yield of 21.6 mg L⁻¹ of SSX2NORD was secreted in the media.

In this study, we have demonstrated that SSX2 is minimally secreted in *P. pastoris* but mainly aggregates in the ER, and it is not translocated to the nucleus. Deletion of NLS from SSX2 improves its folding, thus improving its secretion. Even though the secretion of SSX2NORD is improved, folding inside the ER or post-ER translocation might still be a limiting step for its secretion. Finally, a protocol to produce SSX2NORD in a 2 L fermentor was developed and the secreted SSX2NORD can be used as a DTH skin test reagent of SSX2 cancer vaccine.

Acknowledgment

We would like to thank Dr. Charles K. Barlowe from Dartmouth College for providing the anti-Kar2p for our Kar2p analyses. This project was funded by the Ludwig Institute for Cancer Research.

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CHAPTER 5

PROTEOMIC STUDY OF *PICHIA PASTORIS* CULTURE MEDIA FROM DIFFERENT FERMENTATION CULTURES AND THEIR APPLICATIONS FOR RECOMBINANT PROTEIN PRODUCTION

Abstract

The proteome of *Pichia pastoris* secreted into the culture medium during growth in fermentor was investigated to identify the protein profile and to enhance the purification and secretion of recombinant proteins. Protein samples from culture media and two-dimensional (2D) gel spots were subjected to ESI-MS/MS analysis and searched against a public fungi genome database. A homology MS-BLAST search was also performed to provide information on single peptide hit and unidentified proteins. A total of 34 proteins were identified in different fermentation cultures, including before and after methanol induction, at different temperatures and in host strain overexpressing the recombinant protein Sm-14. In a fed-batch fermentation at 30°C higher protein concentration and more protein species were observed as compared to cultures grown at 25°C. Most proteins identified from culture media from *P. pastoris* have low isoelectric points ($pI < 6$) including naturally secreted proteins and some intracellular proteins. EXG1, Gas1p and some cell wall proteins comprised the majority of the secreted proteins being identified. Intracellular proteins such as alcohol oxidase and peroxiredoxin were also found in culture media from *P. pastoris* induced with methanol. This study demonstrates the identity of proteins in the different fermentation culture media, and suggests potential biotechnology applications based on the discovery of this proteome.

Huang C-J., Damasceno, M. L., Anderson, A. K., Zhang, S., Baumgart, S., Old, J. L., Batt A. C. "Proteomic study of *Pichia pastoris* culture media from different fermentation cultures and their applications for recombinant protein production.

Introduction

A substantial portion of a cellular proteome is defined as the secretome, which ultimately is inclusive of secreted proteins, either anchored on the cell surface or in the extracellular milieu, and the proteins involved in secretory pathway (31). Secreted proteins, both in eukaryotic and prokaryotic cells, perform a wide range of biological functions that are necessary for cells to survive or adjust to environmental changes. These proteins have been implicated in cell wall formation, molecular trafficking, nutrient scavenging, pathogenicity, and defense against competing species (16). The study of secreted proteins can be carried out by directly collecting proteins from the extracellular matrix and analyzing them by mass spectrometry. An alternative method for identifying secreted proteins is to perform a genome-wide computational analysis of the N-terminal peptide of all ORFs for the purpose of predicting secreted proteins (29). However, analysis of the secreted proteome can sometimes be problematic. This is mainly due to the limitations in current proteomic technology, including protein sample preparation, reproducibility of two-dimensional gel electrophoresis, sensitivity of mass spectrometry and completeness of the database utilized (6). Non-secreted proteins may also contaminate a secreted protein preparation because of cell lysis during the cultivation. In addition, the *in silico* method to predict secreted proteins has an inevitable error rate for both false-positive and false-negative predictions as has been reported (16, 30). This is, in part, due to the fact that some secreted proteins are not transported by the conventional ER-Golgi pathway (24), hence, those proteins cannot be identified based upon consensus N-terminal secretion peptide sequence. Nevertheless, scientists are developing new tools and technologies to study the secretome because of its importance in understanding fundamental cellular function, and developing medical and other biotechnology applications (28).

The methylotrophic yeast *Pichia pastoris* has been used successfully in production of various recombinant heterologous proteins from human, animal, plant, fungal, bacterial and viral origins (2). The expressed proteins can be produced either intracellularly or extracellularly. However, secretion of recombinant proteins in *P. pastoris* offers several advantages over other systems. Advantages include a folding pathway that allows disulfide bond formation and post-translational modifications (PTM) which better enables the recombinant proteins to possess their expected biological properties. Secretion of recombinant proteins can also avoid toxicity generated from intracellular accumulation of recombinant proteins. In addition, extracellular production of recombinant proteins is desirable because it simplifies the protein purification process avoiding contamination by all of the intracellular proteins (18). These advantages make secretion a popular route for recombinant protein production in *P. pastoris*. However, *Pichia* also secretes various native proteins into the culture medium, and these native proteins must be removed by purification steps. To advance the use of *P. pastoris*, a detailed profile of host secreted proteins regarding their identities and physical properties can provide critical insights to improve recombinant protein secretion and purification in the *P. pastoris* system. However, there is very little known about *Pichia* secreted proteins despite its use as a recombinant protein production platform for decades.

To produce large quantities of recombinant protein in *P. pastoris*, biomass is allowed to accumulate during fed batch fermentation. In fermentor culture, where the cell growth parameters such as pH, aeration, nutrient and feeding rate can be optimized, *Pichia* reach wet cell weights of over 400 g/L (1). Although several recombinant protein expression vectors driven by different promoters have been constructed for *Pichia*, the *AOX1* promoter is the most widely utilized and successful

system (1,17,18). A two-stage fermentation process, batch and fed-batch, is typically used with strains carrying *AOXI* promoter-driven constructs (32). In the batch phase, cells are cultivated with an *AOXI* promoter repressing carbon source such as glycerol to accumulate biomass. Once the glycerol is depleted, the fed-batch addition of methanol is started to initiate recombinant protein production. To maintain methanol concentration within the tolerance level, three different feeding strategies are normally applied. First, the methanol-feeding rate is controlled according to the methanol concentration in the culture media, indicated by a methanol sensor (4). Second, feeding can be adjusted based on the demand of dissolved oxygen (DO) level in the culture media (DO-state) (20). The third strategy is to control the feeding rate according to the specific growth rate in the induction phase (3). In addition to the methanol feeding strategy, other cultivation parameters including temperature, pH, DO and composition of media have also been reported to affect the yield of recombinant protein production (17). However, the adjustments of the abovementioned parameters to maximize protein production can be protein-specific, and require investigation to identify the crucial parameters for protein production in fermentor cultures.

In this study, we analyzed the *P. pastoris* culture media proteins under different fermentation conditions. The protein profiles of different samples cultivated from the host strain (X-33) and one expressing a recombinant protein (Sm-14) grown at different temperatures, both before induction and after methanol induction, were analyzed. We have identified proteins in these culture media on the basis of isoelectric point (pI) and molecular weight. Information attained from this study will enhance technology related to recombinant protein purification and secretion in *P. pastoris* to expand its biological applications.

Materials and Methods

Strain and plasmids

The wild type strain *Pichia pastoris* X-33 (Invitrogen, Carlsbad, CA) was used in all fermentation experiments. The gene sequence of Sm14 (Genbank Accession no. AY055467.1) with a C62V mutation was acquired from Integrated DNA Technologies, where its genetic codons have been optimized for *P. pastoris*. The Sm14-C64V was cloned into plasmid pPIC α A (Invitrogen) to create plasmid pPIC α ASm14-C64V transformed into competent X-33 strain to create strain Sm-14.

Fed-batch fermentation

A 1.0 mL aliquot of overnight culture of strain X-33 and Sm-14 were used to inoculate 100 mL of BMG medium (100 mM potassium phosphate, pH 6.0, 1.34% (w/v) YNB, 4×10^{-5} % (w/v) biotin and 1% glycerol) and grown at 30°C for 16 hrs until the optical density (OD₆₀₀) reached 20. This culture was used to inoculate the fermentor. The fermentation was carried out in a 2.0 L bench-top fermentor (Bioflo 3000, New Brunswick Scientific, Edison, NJ) with an initial volume of 1.0 L modified basal salts medium (0.23 g L⁻¹, CaSO₄ · 2H₂O, 4.55 g L⁻¹, K₂SO₄, 3.73 g L⁻¹, MgSO₄ · 7H₂O, 1.03 g L⁻¹, KOH, and 6.68 mL L⁻¹, H₃PO₄, 5% (v/v) glycerol and 0.5 mL Antifoam 204 [Sigma, St. Louis, MO]) supplemented with 4.35 mL PTM1 trace salts (24 mM CuSO₄, 0.53 mM NaI, 19.87 mM MnSO₄, 0.83 mM Na₂MoO₄, 0.32 mM boric acid, 2.1 mM CoCl₂, 0.15 mM ZnCl₂, 0.23 M FeSO₄ and 0.82 mM biotin). Ammonium hydroxide (15%) was used as a pH control agent and nitrogen source during the fermentation. AFS-Biocommand Bioprocessing software version 2.6 (New Brunswick Scientific, Edison, NJ) was used for data acquisition and parameter control.

During the fermentation, dissolved oxygen (DO) was maintained at 40% air saturation, and was controlled by a DO cascade of agitation followed by pure oxygen sparging when needed. At the end of the batch phase, indicated by a DO spike, the culture was induced with a methanol feed (100% methanol with 12 mL PTM salts/L). The methanol concentration was controlled at 1 g L⁻¹ and induced for 24 hrs. A methanol sensor, sensor unit and PID control was used to monitor and control methanol concentration.

TCA precipitation

Fermentation samples were collected and centrifuged at 13,000 x g to collect culture supernatant which was filtered through 0.1 µm pore size dead end filter (Pall, East Hill, NY). A total of 2.0 ml of supernatant was pooled and trichloroacetic acid (TCA) was added to a final concentration of 10%, and incubated on ice for an hour. In the case of the 'before induction' fermentation sample, 6.0 ml of supernatant was used. Samples were centrifuged, supernatant was removed, and washed three times with cold acetone. The residual acetone was removed and protein pellets were dried at room temperature for 20 min for further analysis. For 2D gel samples, protein pellets were solubilized in rehydration buffer. For 1 D and in solution samples, 8 M urea was added to resolubilize the protein pellets.

Electrophoresis

Gel electrophoresis with one dimension (1 D)

Different protein pellets were solubilized in 8 M urea and normalized to the same protein concentration before loading on a gel. Protein samples were analyzed by electrophoresis on 12% SDS-polyacrylamide gel (Invitrogen), run at 200 Volt for 1 h. After electrophoresis, the gel was stained by SimplyBlue SafeStain (Invitrogen).

Gel electrophoresis with two dimensions (2D)

The 2D gel electrophoresis was performed by the ZOOM IPGRunner System (Invitrogen). In the first dimension of isoelectric focusing electrophoresis (IEF), protein samples were solubilized in rehydration buffer (2 M thiourea, 5 M urea, 0.8% carrier ampholytes (pH 4-7), 2% CHAPS, and 20 mM DTT) and added into the strip cassette to rehydrate the 7 cm immobilized pH gradient strips (IPG strip pH 4-7, Invitrogen) for 16 hrs. The IEF was carried out with the following parameters: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000 V for 120 min. After IEF, the strips were equilibrated (1X LDS and 50 mM DTT) for 15 min, alkylated (1X LDS and 125 mM iodoacetamide) for another 15 min, then placed on top of a 4% to 12% gradient SDS polyacrylamide gel (Invitrogen) and sealed with 0.5% agarose. The gels were run at 75 V for 10 min and then 200 V for another 40 min, and stained by SimplyBlue SafeStain (Invitrogen). A total of 28 spots from three 2D gels were excised by scalpel blade and transferred to 0.6 ml tubes for protein identification analysis.

Protein concentration and wet cell weight

An aliquot of 1.0 ml of fermentation broth was sampled and centrifuged at 13,000 x g for 10 min. The supernatant was collected for protein concentration analysis. Total protein concentration was determined by a Coomassie (Bradford) protein assay kit (Pierce Biotechnology, Rockford, IL) by using different concentrations of bovine serum albumin (BSA) as standards. 10 to 30 μ L of each protein sample was added to a 96 well plate and each well received 295 μ L of Coomassie reagent. After incubation at room temperature for 10 min, the absorbance at 595 nm was collected by a TECAN GENios microplate reader (TECAN group Inc., Mannedorf, Switzerland). The protein concentration of each sample was determined

according to a BSA standard curve. To determine wet cell weight (WCW), after supernatant was removed, the cell pellet was weighed to determine WCW.

Sample preparation for mass spectrometry analysis

A total of 28 gel plugs were subjected to in-gel digestion by trypsin and the resultant digested peptides were extracted for mass analysis as described by Shevchenko *et al.* (27) with slight modifications. After digestion, the supernatant was collected and a total of two extractions on a gel plug were performed. First was 30 μ L of 50% acetonitrile (ACN) with 5% formic acid (FA) for 45 min followed by 30 μ L of 90% ACN with 5% FA for 5 min. All supernatants were combined and dried in a SpeedVac concentrator (Thermo Savant, Holbrook, NY). For in solution samples, 5 μ g protein of each sample were collected and subjected to reduction and alkylation in 10 mM DTT and 55 mM iodoacetamide respectively. Protein samples were digested with trypsin overnight, added with 20 μ L FA and desalted by C18 solid phase extraction (SPE) columns. All samples were then dried in a SpeedVac concentrator for further analysis.

Reversed phase HPLC and tandem mass spectrometry (LC-MS/MS)

The peptide samples were reconstituted with 10 μ L of 2% ACN with 0.5% FA. A total of 6.4 μ L were injected onto a PepMap C-18 trap column (5 μ m, 300 μ m id \times 5 mm, Dionex) and separated on a PepMap C-18 RP Nano column (3 μ m, 75 μ m id \times 150 cm, Dionex), using a 30-60 min gradient of 5% to 45% ACN in 0.1% FA at 275 nL/min to elute the peptides. The NanoLC was connected in-line to a hybrid triple quadrupole linear ion trap mass spectrometer, 4000 Q Trap (Applied Biosystems, Foster City, CA) equipped with a nano ion source. The ESI-MS/MS data acquisition was performed using the analyst 1.4.2 software in the positive ion mode for

information-dependent acquisition (IDA) analysis. The nanospray voltage was set at 2.0 kV for all experiments with nitrogen gas used as both the curtain gas and collision gas that the heated interface was set at 150 °C . The declustering potential was set 50 eV to minimize in-source fragmentation. The ion source nebulizer (gas 1) was set to 20 (arbitrary unit). For IDA analysis, after each survey scan from m/z 400 to 1600, an enhanced resolution scan was carried out with multiple charge states. Rolling collision energy based on different charged states and m/z values was used to obtain optimal MS/MS spectra.

Data analysis and protein identification

The nanoLC-ESI based IDA files used for database searching by MASCOT 2.2 to search against the NCBI fungi database downloaded in January 2009. The search parameters allowed for one miscleavage, and variable modifications of methionine oxidation and cysteine carboxyamidomethylation with a peptide tolerance of 1.5 Da, and MS/MS tolerance of 0.6 Da for LC-SEI. All matches were above a 95% confidence interval (CI) with significant scores defined by MASCOT probability analysis (www.matrixscience.com/help/scoring_help.htm#PBM) greater than “identity” considered confidentially-hit peptides and used for protein identifications. The IDA raw data files were also subjected to MS spectra-based blast homology search. The MS BLAST Homology search was carried out by ProBlast search software 1.4 (Applied Biosystems, Foster City, CA) for all spots to determine the protein homologs in the *S. cerevisiae* protein database downloaded in April 2009 (25). For those peptide hit and unidentified spots, the raw mass spectra were first searched against the NCBI *Schistosoma* database download in April 2009 to eliminate Sm14-related spots. For the remainder of the protein spots, the top hit of the homolog search is shown.

Results

Preliminary analysis of culture media proteins in different fermentation cultures

The optimum growth temperature for *P. pastoris* has been reported at 28 to 30 °C. However, some studies have shown that decreasing temperature below 25 °C can significantly improve recombinant protein productivity without hampering cell growth (4, 7). To understand the cell growth kinetics and profile of proteins in culture media the *P. pastoris* host strain and derivatives expressing SM-14, several fermentations were performed at different cultivation temperatures and their fermentation profiles were compared. The cell growth, extracellular protein concentration, and methanol utilization of X-33 at 30°C and 25°C, and Sm14 at 25°C are shown in Figure 5-1. When X-33 grew at 30°C, the batch phase was 22 hrs and WCW reached 410 mg mL⁻¹ after methanol induction for 24 hrs. The amount of consumed methanol was 695 g and the protein concentration in the media was 0.34 mg mL⁻¹ where both values were higher than X-33 cultivated at 25°C (477 g and 0.105 mg mL⁻¹, respectively). These data indicated that cells utilized more methanol at 30°C at induction phase. For the Sm14 strain, the WCW and methanol consumption rate were 0.38 mg mL⁻¹ and 341 g, respectively, which were lower than that observed for X-33 cultivated at 25°C, suggesting that expression of Sm14 C64V affects cell growth and methanol consumption. However, the average media protein concentration was 0.145 mg mL⁻¹, 0.03 mL⁻¹ mg higher than that observed for the X-33 strain grown at 25°C. The SDS-PAGE analysis of protein samples, for both ‘before induction’ and ‘after induction for 24 hrs’ of three fermentations is shown in Figure 5-2. Protein species in the samples from the ‘before induction’ fermentations did not show obvious differences. However,

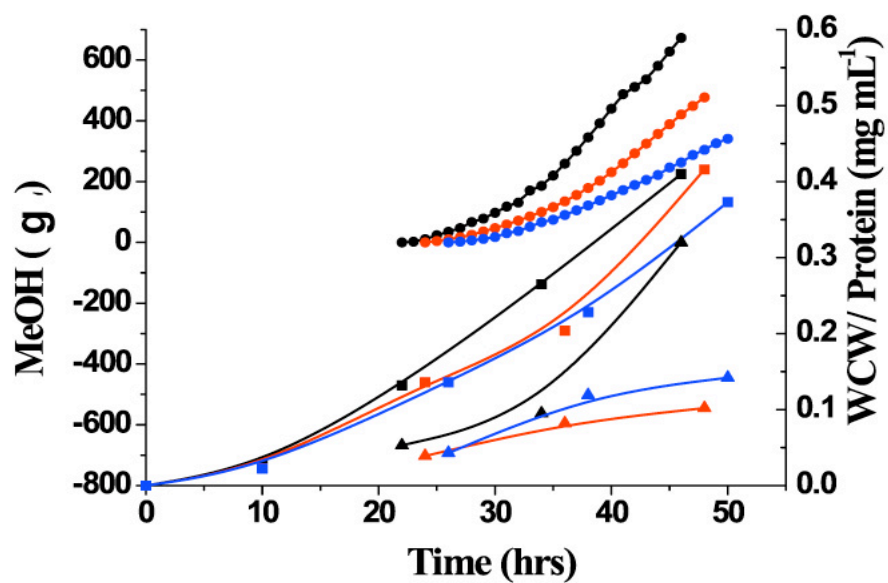


Figure 5-1. Growth curves, culture media protein concentrations and consumed methanol of strain X-33 and Sm14 cultivated in different conditions. Black line, X-33 at 30°C; Red line, X-33 at 25°C; Blue line, Sm14 at 25°C. Circle, methanol volume; square, Wet Cell Weight (WCW); Triangle, culture media protein concentration.

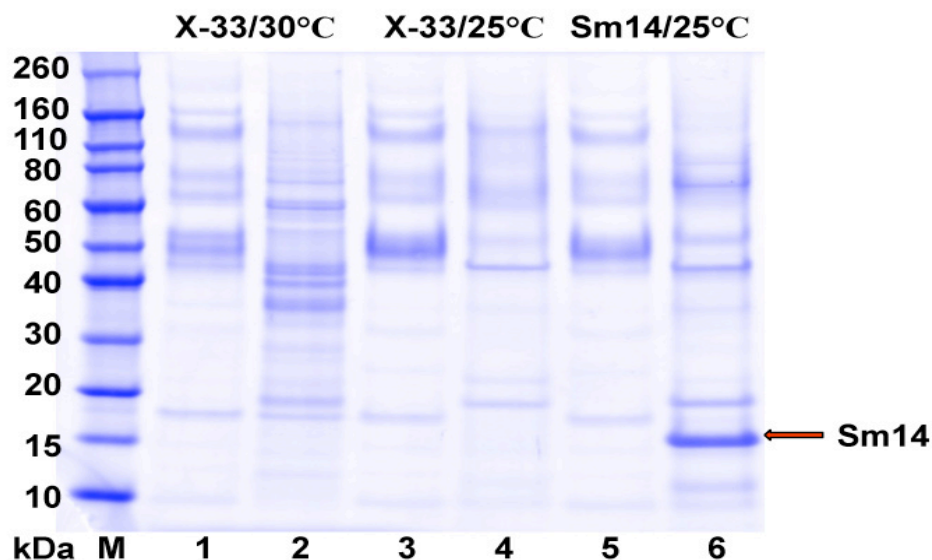


Figure 5-2. SDS-PAGE analysis of different fermentation protein samples from X-33 and Sm14 strains before (lane 1,3, and 5) or after induction for 24 hrs (lane 2,4 and 6). M, molecular weight marker. Each lane was loaded with 4 μ g of protein.

Table 5-1. Proteins identified in *P. pastoris* X-33 before induction with methanol at 25°C

No.	Accession No.	Protein I.D.	Score	Mr(kDa)/pI	Coverage (%)
1	gi 13235614	protein disulphide isomerase [<i>Pichia pastoris</i>]	270	57.7/4.59	43.9
2	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	230	47.8/4.53	32.9
3*	gi 50295066	hypothetical protein [<i>Candida glabrata</i> CBS138]	199	38.7/4.96	3.8
4*	gi 90655997	Gas1p [<i>Pichia pastoris</i>]	158	57.3/4.02	12.5
5*	gi 33316725	26S proteasome subunit [<i>Paracoccidioides brasiliensis</i>]	48	46.4/7.29	3.1
6*	gi 85096882	hypothetical protein [<i>Neurospora crassa</i> OR74A]	48	96.9/7.04	2.9
7*	gi 71021175	hypothetical protein [<i>Ustilago maydis</i> 521]	47	214.8/7.52	0.5

* Single peptide hit

Table 5-2. Proteins identified from the culture medium of X-33 cultivated at 25°C after methanol induction for 24 hrs.

No.	Accession No.	Protein I.D.	Score	Mr(kDa)/pI	Coverage (%)
1	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	260	47.8/4.53	31.4
2	gi 90655997	Gas1p [<i>Pichia pastoris</i>]	157	57.3/4.02	11.5
3	gi 145369574	Fet3 protein [<i>Pichia pastoris</i>]	87	70.8/4.21	10.5
4*	gi 19114970	ribomal-ubiquitin fusion protein Ubi5 [<i>Schizosaccharomyces pombe</i> 972h-]	86	17.2/9.78	27.3
5*	gi 1709725	Acid phosphatase PHO1 precursor [<i>Pichia pastoris</i>]	66	52.7/4.41	3.8
6*	gi 119501228	conserved hypothetical protein [<i>Neosartorya fischeri</i> NRRL 181]	59	51.7/8.35	1.8

* Single peptide hit

Table 5-3. Proteins identified from the culture medium of Sm14 cultivated at 25°C after methanol induction for 24 hrs.

No.	Accession No.	Protein I.D.	Score	Mr(kDa)/pI	Coverage (%)
1	gi 2104961	alcohol oxidase [<i>Pichia pastoris</i>]	482	73.9/6.04	43.3
2	gi 90655997	Gas1p [<i>Pichia pastoris</i>]	193	57.3/4.02	10.6
3	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	180	47.8/4.53	33.3
4	gi 47132400	methionine synthase [<i>Pichia pastoris</i>]	123	85.9/5.84	8.1
5*	gi 115433100	nucleoside diphosphate kinase [<i>Aspergillus terreus</i> NIH2624]	93	16.9/8.52	11.1
6*	gi 2641213	polyubiquitin [<i>Schizophyllum commune</i>]	70	15.6/5.87	24.5
7*	gi 145369574	Fet3 protein [<i>Pichia pastoris</i>]	68	70.9/4.21	1.7
8*	gi 50295066	hypothetical protein [<i>Candida glabrata</i> CBS138]	63	38.7/4.96	3.8
9*	gi 154323902	enolase [<i>Botryotinia fuckeliana</i> B05.10]	63	47.1/5.26	3.4
10*	gi 50556202	hypothetical protein [<i>Yarrowia lipolytica</i>]	62	47.3/5.01	4.1
11*	gi 146415024	conserved hypothetical protein [<i>Pichia guilliermondii</i> ATCC 6260]	62	35.6/5.44	7.8
12*	gi 50546891	hypothetical protein [<i>Yarrowia lipolytica</i>]	57	21.4/5.25	16.8
13*	gi 71018545	hypothetical protein [<i>Ustilago maydis</i> 521]	51	47.5/5.37	2.2

* Single peptide hit

Table 5-4. Proteins identified from the culture medium of X-33 cultivated at 30°C after methanol induction for 24 hrs.

No.	Accession No.	Protein I.D.	Score	Mr(kDa)/pI	Coverage (%)
1	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	320	47.8/4.53	33.1
2	gi 1709725	Acid phosphatase PHO1 precursor [<i>Pichia pastoris</i>]	238	52.7/4.41	34.4
3	gi 2104961	alcohol oxidase [<i>Pichia pastoris</i>]	158	73.9/6.04	37.4
4*	gi 146418172	peroxiredoxin TSA1 [<i>Pichia guilliermondii</i> ATCC 6260]	115	21.7/4.96	9.2
5*	gi 119501228	conserved hypothetical protein [<i>Neosartorya fischeri</i> NRRL 181]	71	51.7/8.35	1.8
6*	gi 121700579	conserved hypothetical protein [<i>Aspergillus clavatus</i> NRRL 1]	55	50/5.11	2.4
7*	gi 6322068	Sim1p [<i>Saccharomyces cerevisiae</i>]	55	48/4.47	2.5
8*	gi 71004110	hypothetical protein [<i>Ustilago maydis</i> 521]	54	18.5/6.24	2.1
9*	gi 71003950	hypothetical protein [<i>Ustilago maydis</i> 521]	52	153/5.93	2.2
10*	gi 231690	Peroxisomal catalase [<i>Pichia angusta</i>]	52	57.8/6.42	4.7
11*	gi 45190496	AEL111Cp [<i>Ashbya gossypii</i> ATCC 10895]	51	35.7/5.02	2.3
12*	gi 146414696	hypothetical protein [<i>Pichia guilliermondii</i> ATCC 6260]	49	97.6/5.73	0.9
13*	gi 149238159	conserved hypothetical protein [<i>Lodderomyces elongisporus</i> NRRL YB-4239]	48	48.4/7.33	3.8
14*	gi 3047316	ubiquitin fusion protein [<i>Magnaporthe grisea</i>]	47	17.3/9.79	39.6
15*	gi 150864141	hypothetical protein [<i>Pichia stipitis</i> CBS 6054]	47	24.4/4.8	5.1
16*	gi 169860983	hypothetical protein [<i>Coprinopsis cinerea</i> okayama7#130]	47	44.5/9.22	8.1
17*	gi 121707662	phosphotransmitter protein Ypd1 [<i>Aspergillus clavatus</i> NRRL 1]	46	18.9/4.81	4.8
18*	gi 71021421	hypothetical protein [<i>Ustilago maydis</i> 521]	46	40.7/6.27	4

* Single peptide hit

for the ‘after induction’ samples, the protein species were quite different among three different conditions, particularly a diversified protein profile was found in cells cultivated at 30°C. To identify proteins present in culture media, four protein samples, three ‘after induction’ samples and one ‘before induction’ sample (X-33 at 25°C), were collected, TCA precipitated, solubilized in 8 M urea and digested with trypsin. The resulted peptides were later separated and analyzed by nano-LC ESI-MS/MS. By searching against the NCBI fungi protein database, seven protein species were identified in the ‘before induction’ condition (Table 5-1). When both X-33 and Sm14 strains were cultivated at 25°C, there were six and 13 protein species detected, respectively (Table 5-2 and 5-3). From the culture media of X-33 cultivated at 30°C (Table 5-4), 18 protein species were identified, which correlated with the observation from SDS-PAGE that it had a greater number of protein species. However, both high protein concentration and complex protein species in the culture medium at 30°C cultivation indicated that this is not a favorable fermentation condition for production of recombinant protein from the objective of protein purification. Therefore, samples of X-33 and Sm14 cultivated at 25°C were collected to further identify and characterize proteins in the respective culture media by using 2D gel electrophoresis.

2D gel analysis of culture media proteins

Samples of X-33 and Sm14 cultivated at 25°C were TCA precipitated, solubilized in rehydration buffer and loaded on IPG strips to perform 2D gel electrophoresis. Preliminary investigations using pH 3-10 strips showed that pI's of most culture media proteins were less than 7.0. To have a better resolution of protein spots, pH 4-7 strips were used in this study. As shown in Figure 5-3, the vast majority of culture media proteins exhibited pI less than 6.0 with molecular weight ranging from 10 to 260 kDa. A total of 28 protein spots were excised from three gels, digested

with trypsin, analyzed by Nano/LC ESI-MS/MS and a Mascot search against the NCBI fungi database was performed to determine the protein identity of each spot. Out of 17 spots, there was identity with proteins that have both a high protein score, and at least two peptide hits indicating high confidence of the identified proteins (Table 5-5). A total of six spots had only single peptide hit proteins and five spots failed to yield any identity. As shown in Figure 5-3 and Table 5-5, naturally secreted proteins, such as exo-beta-1,3-glucanase, Gas1p and acid phosphatase, were found in the culture supernatant, especially exo-beta-1,3-glucanase was presented in 12 excised spots. However, intracellular proteins such as alcohol oxidase and peroxiredoxin were also identified in the culture media of post-methanol induction samples. These proteins are related to methanol metabolism, including methanol utilization and protective responses to oxidants generated by methanol oxidation. Protein disulfide isomerase and enolase, which are proteins present in both the intracellular and extracellular milieu, were also identified. Since most spots were cut from the Sm14 culture media gel, some spots might be Sm14 or its degradation products. Therefore, a MASCOT search against the NCBI *Schistosoma* protein database was performed for all protein spots in order to identify Sm14-related spots. Spots numbered 18, 21, 25 were identified as Sm 14 with multiple peptide hits and a high protein score. In total, there were eight protein spots, four with one single peptide hit and four unidentified, requiring further characterization. It should be note that manual inspection of all raw data files which yielded either single peptide hit or no hit found numerous high quality MS/MS spectra from multiply-charged precursor ions, suggesting the failure of identification for those 2D spot samples is likely due to either no equivalent genomic database available or unexpectedly heavy modifications.

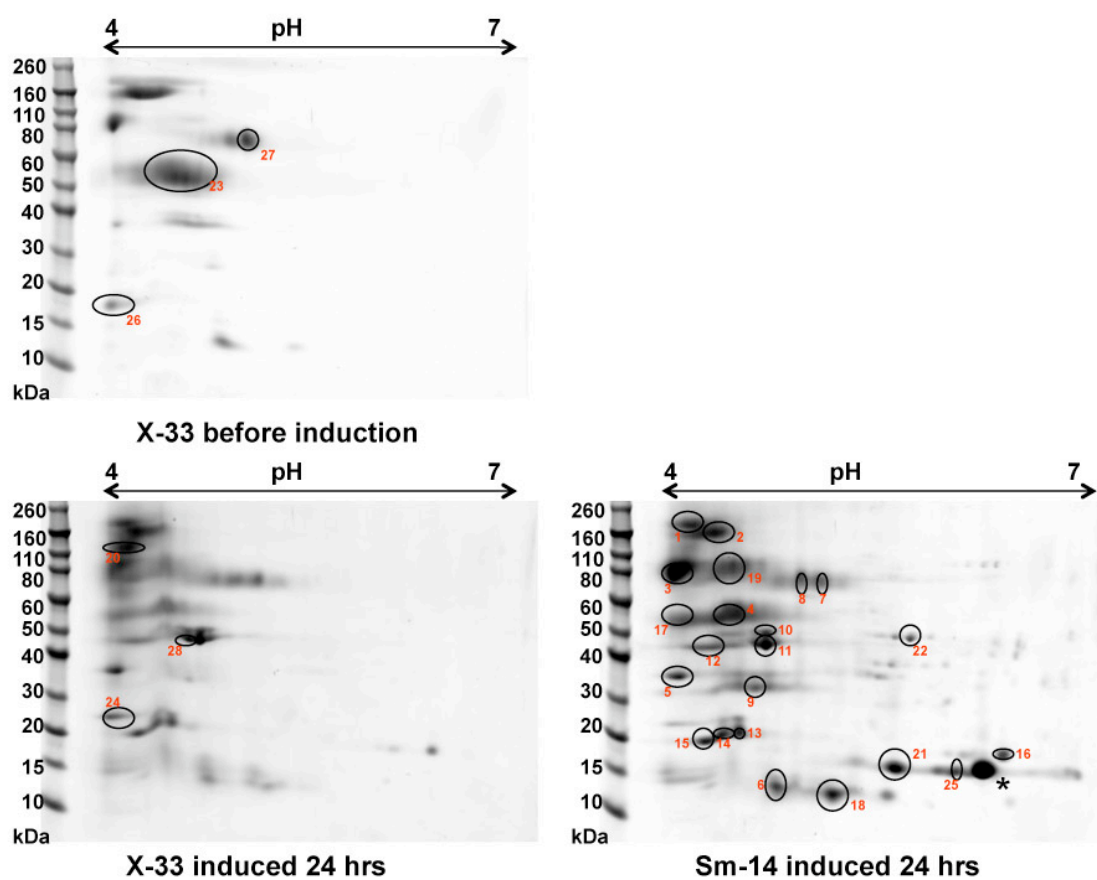


Figure 5-3. 2D gel images of different samples prepared from culture media of X-33 and Sm14 strains. In total twenty-eight spots were selected for Nano/LC-MS/MS analysis. * Sm14

Table 5-5. Protein identification of seventeen 2D gel spots of more than two peptide hits.

Spot No.	Accession No.	Protein I.D.	Score	Mr(kDa)/pI	Coverage (%)
1	gi 90655997	Gas1p [<i>Pichia pastoris</i>]	102	57.3/4.02	4.6
	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	72	47.8/4.53	6
2	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	162	47.8/4.53	14.5
3	gi 90655997	Gas1p [<i>Pichia pastoris</i>]	266	57.3/4.02	15.1
	gi 1709725	Acid phosphatase PHO1 precursor [<i>Pichia pastoris</i>]	80	52.6/4.41	4.1
	gi 63054403*	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	58	47.8/4.53	8
4	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	90	47.8/4.53	8
	gi 71064095*	Sim1p [<i>Saccharomyces cerevisiae</i>]	59	46.4/4.46	4.4
7	gi 2104961	alcohol oxidase [<i>Pichia pastoris</i>]	167	73.8/6.04	22.6
8	gi 2104961	alcohol oxidase [<i>Pichia pastoris</i>]	287	73.8/6.04	31.4
10	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	150	47.8/4.53	24.2
	gi 13235614*	protein disulphide isomerase [<i>Pichia pastoris</i>]	50	57.8/4.59	3.9
11	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	137	47.8/4.53	19.3
12	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	377	47.8/4.53	37.2
13	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	238	47.8/4.53	22.7
14	gi 146418172	peroxiredoxin TSA1 [<i>Pichia guilliermondii</i> ATCC 6260]	52	21.7/4.96	13.3
15	gi 63054403*	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	78	47.8/4.53	8.5
	gi 146418172	peroxiredoxin TSA1 [<i>Pichia guilliermondii</i> ATCC 6260]	57	21.8/4.98	13.3
17	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	73	47.8/4.53	14.3
19	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	259	47.7/4.53	31.4
	gi 2104961	alcohol oxidase [<i>Pichia pastoris</i>]	102	73.8/6.04	11
	gi 145369574*	Fet3 protein [<i>Pichia pastoris</i>]	101	70.9/4.21	6
	gi 1709725*	Acid phosphatase PHO1 precursor [<i>Pichia pastoris</i>]	63	52.7/4.41	5.8
	gi 90655997	Gas1p [<i>Pichia pastoris</i>]	276	57.3/4.02	15.1
20	gi 63054403	exo-beta-1,3-glucanase [<i>Pichia pastoris</i>]	238	47.8/4.53	26.6
	gi 38146742*	aspartic protease yapsin 1 [<i>Pichia pastoris</i>]	53	63.0/4.48	1.5
	gi 145369574*	Fet3 protein [<i>Pichia pastoris</i>]	48	70.9/4.21	3.9
	gi 50556202	hypothetical protein [<i>Yarrowia lipolytica</i>]	162	47.4/5.01	10.5
22	gi 58264446*	phosphopyruvate hydratase [<i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21]	104	47.6/5.38	4.3
	gi 13235614	protein disulphide isomerase [<i>Pichia pastoris</i>]	156	57.8/4.59	17.4

*Single peptide hit protein of *P. pastoris*

Homology search of single peptide hit and unidentified protein spots

To provide further information about single peptide hit and unidentified spots (low-identity spots) from previous 2D gels, the MS spectra of all protein spots were submitted for MS BLAST searches against the *S. cerevisiae* protein database. For low-identity spots, seven out of eight protein spots had homologs in *S. cerevisiae*, and the top hit protein of each protein spot is shown in Table 5-6. Spot 16 was identified as a superoxide dismutase by MASCOT and as PMR1 protein, a $\text{Ca}^{+2}/\text{Mn}^{+2}$ binding ATPase, by homolog search. Both are metal chelating proteins with different cellular functions. Spots numbered 24, 26 and 28 all had a single peptide hit of a hypothetical protein of *Candida glabrata*. The homolog search identified these spots as Scw4p and Scw10p which belong to a class of soluble cell wall proteins with similarity to glucanases. For unidentified spots, spot five had a top hit with Sun4p protein which is also a cell wall protein with possible function in cell wall septation. Spots of nine and 20 were identified as Sim1p and Ino80p, respectively, and both proteins were indicated as intracellular proteins by their cellular functions.

Discussion

One of the major limitations in proteomic research is the availability and completeness of the protein database. To quickly and correctly identify a protein of interest, a comprehensive database is required (9, 15). Nevertheless, a proteomic study of an unsequenced organism such as CHO cells could still be carried out by searching respective MS spectra against databases of rodent, human or all species (19). Although the genome of *P. pastoris* was sequenced, the database is not free accessibility to the public (5). Therefore, in this study, a MASCOT search of all MS spectra generated

Table 5-6. MS BLAST search of low confidence 2D gel spots to identify protein homologs in *Saccharomyces cerevisiae*.

Spot No.	Identified by MASCOT to fungi database			Identified by MS BLAST to <i>S. cerevisiae</i>	
	Accession No.	Protein I.D.	Mr(kDa) /pI	Protein I.D. of top hit	Mr(kDa)/pI
16	gi 37704551*	Superoxide dismutase [CB]	14.8/5.93	PMR1, high affinity Ca ²⁺ /Mn ²⁺ P type ATPase	104.5/5.45
	gi 6094316*	Superoxide dismutase [CA]	16.1/5.68		
24	gi 50295066*	Hypothetical protein [CG]	38.7/4.96	Scw4p, cell wall protein with similarity to glucanases	40.2/4.52
	gi 119501228*	Conserved hypothetical protein [NF]	51.7/8.35		
26	gi 50295066*	Hypothetical protein [CG]	38.7/4.96	Scw4p, cell wall protein with similarity to glucanases	40.2/4.52
28	gi 50295066*	Hypothetical protein [CG]	38.7/4.96	Scw10p, cell wall protein with similarity to glucanases	40.5/4.32
	gi 63054403*	Exo-beta-1,3-glucanase [PP]	47.8/4.53		
5	n.i.			Sun4p, cell wall protein related to glucanases; possibly involved in cell wall septation	43.4/4.02
6	n.i.			No positive hit	
9	n.i.			Sim1p, protein of the SUN family that may participate in DNA replication and may be cell cycle-regulated	48.1/4.31
27	n.i.			Ino80p, inositol requiring protein	171.5/5.54

Abbreviation of organisms: CB, *Cryptococcus bacillisporus*; CG, *Candida glabrata*; CA, *Candida albicans*; NF, *Neosartorva fischeri*; PP, *Pichia pastoris*; n.i.: no identification. * single peptide hit

from the solution samples and the 2D gel spots were performed against the public NCBI fungi database which also contains a portion of the *P. pastoris* protein sequences. A detailed analysis of 2D gel spots further provided a better resolution of culture media proteins and showed their characteristics such as abundance, pI and molecular weight. To complement database weakness, a homolog search of MS spectra from all samples against the *S. cerevisiae* protein database was performed to provide additional information for low-confidence protein spots. Without access to an available completed *P. pastoris* protein database, our study provides a novel discovery of the proteome in *P. pastoris* culture media, under different fermentation conditions.

In the preliminary study, protein concentrations and profiles of before induction samples were similar among the different fermentation conditions (Figures 5-1 and 5-2). After transition to the methanol-consumption phase, protein concentration and protein species increased in Sm14-expression and 30°C cultures. Particularly cultivation at 30°C has been shown to increase cell lysis and proteolytic activity (11). From the MS data of solution samples, alcohol oxidase and other intracellular proteins were detected in culture media of the 30°C and Sm14-expressing cultures, indicating cell lysis after methanol induction. Several native secreted proteins, such as beta-1,3-glucanase, Gas1p, acid phosphatase and Fet3, were identified in different culture media, particularly, beta-1,3-glucanase was found in all samples. Surprisingly, protein disulfide isomerase (PDI) was detected in the culture media of the before induction sample and found to be the dominant protein. PDI is occasionally found in yeast culture media and is mainly located at the endoplasmic reticulum (23, 29). Even though we have identified a total of 34 proteins in the different fermentation culture media, the number of identified proteins is less when

compared to a similar study conducted by Swain *et al.*, which have identified 81 protein species in the secretome of *Kluyveromyces lactis* (29). This could be attributed to a different sample preparation protocol, incomplete *P. pastoris* database, or in reality, *P. pastoris* secretes lower levels of native proteins (18).

It is known that the optimal temperature for recombinant protein production in *P. pastoris* is protein-specific and related to other physiological conditions such as pH of the media (12). When X-33 was cultivated at 30°C with pH 6.0 in a fed-batch fermentation, the extracellular protein concentration reached 0.34 mg mL⁻¹, three times higher than that observed in the 25°C cultures. A similar experiment conducted by Hong *et al.*, also found that cultivation at 30°C exhibited much higher protein concentration in the culture media than cultures at 25°C (10). Regardless of temperature effect on recombinant protein production, high concentration of host proteins in the culture media represents a great challenge for recombinant protein purification. These host proteins are considered as impurity contaminants and must be removed during the purification process (33). In addition, SDS-PAGE and MS analysis have shown that there were more diversified protein species in the culture media of the 30°C cultivation (Figure 5-2 and Table 5-4). Expression of Sm14 at 30°C also revealed high host protein concentration in the culture media (data not shown). These results suggested that *P. pastoris* fermentation carried out at 30°C is not a favored condition, especially for the objective of recombinant protein purification. Therefore, a more comprehensive 2D gel proteome analysis was carried out for the 25°C cultures.

From the 2D gel analysis of culture media samples, it was very interesting that most proteins have acidic pI. A study of the secretome of *Pleurotus sapidus* revealed that the majority of secreted proteins are metallopeptidases and serine proteases that

also have low *pI* (36). In *P. pastoris*, however, the identified proteins were more diversified including secreted and intracellular proteins. Some protein spots were identified as the same protein with identical molecular weight but with different *pI*. This could be a common problem in 2D gel electrophoresis or posttranslational modification of the protein (5). Among the secreted proteins, exo-beta-1,3-glucanase was identified in 12 of the 28 spots. These 12 spots showed high protein scores and exhibited multiple peptide hits indicating the presence of exo-beta-1,3-glucanase. Among the 12 spots, spot 12 had the highest protein score and displayed a reasonable molecular weight (47.8 kDa) and *pI* (4.53) characteristic of exo-beta-1,3-glucanase. Other spots may also be exo-beta-1,3-glucanase that shifted their molecular weights and *pI*s due to post-translational modification (glycosylation) and degradation. Although research has shown that exo-beta-1,3-glucanase of *P. pastoris* is not a glycoprotein, most of the yeast exoglucanases have proven to be glycosylated (8, 34). Hence, a glycostain was done, revealing that most of the higher molecular weight spots contained glycoproteins, including spots two, four, 17, 19 and 20 which were identified as exo-beta-1,3-glucanase (data not shown). Therefore, it is possible that the 12 spots of exo-beta-1,3-glucanase are the non-glycosylated form, glycosylated form or the degradation fragments of both. Intracellular proteins, including alcohol oxidase, peroxiredoxin, and nucleotide diphosphate kinase, were detected in the culture media of after induction samples. The presence of these intracellular proteins was due to cell lysis, especially alcohol oxidase can account for over 30% of intracellular proteins in cultures using methanol as the carbon source (13). The secretome was defined as the proteome of the secreted proteins, and the cellular machinery involved in their secretion (14). Therefore, this study provides a novel discovery and survey of the proteome with a greater range than just the secretome: a real-time analysis of the proteins in the fermentation culture media.

Homology-driven proteomics is used to explore the proteome of organisms with unsequenced genomes to provide reliable sequence similarity identification of unknown proteins (26). The so-called MS BLAST approach is based on a unique search algorithm which uses direct raw IDA data files as input spectra to perform homolog search against the designated database instead of traditional “complete match” database search approach. This MS BLAST strategy has been used to identify proteins of *P. pastoris* by homolog search against the *S. cerevisiae* genome database: 15 out of 19 protein bands were successfully identified (25). In this study, that approach was used to find *S. cerevisiae* homologs of single peptide hit and unidentified protein spots. Two cell wall proteins (CWPs), Scw4p and Scw10p, linked to the cell wall via an alkaline-sensitive linkage were identified. These two proteins are related to glucanases with possible functions in mediating cell-cell interactions and cell wall biogenesis (35). It is not a surprise to find them present in the culture media. Sun4p and Sim1p were also identified and both belong to the SUN family of *S. cerevisiae*. In the SUN family, genes share 75% to 85% identity of 258 amino acids at their C-terminus, but appear to be involved in different cellular functions (22). Sim1p is reported to participate in DNA replication and Sun4p is described as a cell wall protein involved in cell septation (21). Using this strategy, seven out eight spots were successfully identified to provide supplemental information to the proteome of the fermentation culture media.

Understanding the proteome of the fermentation culture media is important to recombinant protein purification and secretion in *P. pastoris*. First, fermentation is the protocol widely used for recombinant protein production. This proteome shows a real-time host protein profile presented in the culture media. Second, protein characteristics, including molecular weight, pI, hydrophobicity, of media proteins can

be revealed which can aid recombinant protein purification. For example, if the secreted recombinant protein has a basic *pI* value, anion exchange chromatography would be a good choice for the first capture step. Third, several secreted proteins were identified and the use of these secreted proteins as fusion protein partners or their secretion signals can build a tool box to improve recombinant protein secretion. Collectively, this proteome data expands the biotechnology applications of *P. pastoris*.

In this study, we have examined the proteome of the culture media of *P. pastoris* under different fermentation conditions. A total of 34 proteins were directly identified from solutions of the different culture media. Spots analysis from 2D gels allowed identification of more proteins, and revealed some of their characteristics of this proteome. These data will enhance methods of recombinant protein secretion and purification in *P. pastoris*. However, the analysis of the proteome is far from complete due to the lack of the sequenced *P. pastoris* genome. Once the genome sequence become public, we are hoping to identify more proteins from our techniques to reveal more details about this proteome.

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CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

In this study, two popular recombinant protein expression systems, *Escherichia coli* and *Pichia pastoris*, have been used to produce cancer vaccine SSX2 and its DTH skin test reagent. In fact, initial work to secrete SSX2 in *P. pastoris* to take advantage of its folding and secretion pathway to simplify its purification process and to avoid contamination of intracellular proteins (9). Various studies have been performed to improve its secretion, such as cultivation at different conditions, overexpression ER chaperones and foldases, and utilization of different secretion signals. These conditions did not significantly improve SSX2 secretion. In addition, one of the goals of this study is to produce SSX2 for its proof-of-principle clinical trial which requires high protein yield in order to generate sufficient amount of vaccine for cancer patients (7). Therefore, *E. coli* was chosen as expression host for its high yield expression of SSX2 and its production and purification process were also developed (8).

SSX2 was purified from *E. coli* inclusion bodies and solubilized in urea. Like other cancer vaccines such as NY-ESO-1 and MAGE-A3, it will be formulated in 4M urea and the unfolded SSX2 will be used as a cancer vaccine (3, 10). This is due to the fact that Major Histocompatibility Complex (MHC) class one epitopes are generated from the degraded peptides, therefore there is unnecessary to maintain the biological activity of cancer antigen (11). The therapeutic strategy is to deliver SSX2 to Antigen Presenting Cells (APC), for example Dendritic Cells, to induce and activate SSX2-specific cytotoxic T lymphocytes to target and kill the cancer cells through a process called cancer immunotherapy (12, 13). Several studies have demonstrated that SSX2 can elicit both humoral and cellular (both CD4⁺ and CD8⁺) mediated immune

responses to kill cancer cells displaying SSX2 epitopes on cell surface (1, 2). Therefore, there is an urgent need to scale up the process to produce SSX2 for the proof-of-principle trials.

For the first part of this work, due to its successful expression *in E. coli* C41 (DE3) strain, the production of SSX2 was scaled-up in a 2L fermentor equipped with a fed-batch protocol using a pH-state control to reach the high cell density of 260 mg/ml and protein yield of 1105 mg/L. The purification method, including cell lysis, tangential flow filtration and three chromatographies, effectively removes *E. coli* contaminants from SSX2 antigen. Among all the purification steps, hydrophobic interaction chromatography was most effective regarding both contaminant and endotoxin removal. This is due to the fact that SSX2 is a predicted hydrophilic protein and endotoxin is characteristically hydrophobic due to its lipid A tail (16). Even though this study provides a reasonable yield for SSX2 production, the expression of SSX2 seems to be toxic according to the dissolved oxygen (DO) profile during the fermentation, a sharp increase in DO after protein induction. Experiments to optimize SSX2 expression and cell growth, such as induction time, IPTG concentration and different cultivation temperatures, can be carried to avoid the toxicity effect of SSX2 expression and maximize its production. In addition, although the study also shows that the purified SSX2 can be recognized by the SSX2-antibodies from the sera of the cancer patients, the preliminary *in vitro* or *in vivo* experiments to show the activation of SSX2-specific cytotoxic T lymphocytes by the purified SSX2 can be performed to demonstrate its anti-cancer immune responses.

Process development was later studied to improve the protein yield, purity and endotoxin level of the SSX2 antigen, and the new process was scaled up to a 20L fermentor in the Cornell/Ludwig Institute for Cancer Research cGMP Facility.

Phosphate buffer was found to improve SSX2 solubility, therefore increasing the protein recovery during the anion exchange process. A fourth chromatography, Q sepharose XL (Q XL), was introduced to specifically remove endotoxin and to improve the protein purity. The new process, incorporating phosphate buffer and an additional Q XL column, was scaled-up and produced 420 mg of SSX2 with endotoxin level less than 0.04 EU/ μ g and purity of over 97%. The urea-solubilized SSX2 was refolded for a potential use as another format of SSX2 cancer vaccine that can activate T helper cells ($CD4^+$) to promote the growth and memory maintenance of SSX2-cytotoxic T lymphocytes (5). Even though, the scaled-up process produced high quality of SSX2, low protein yield was found in the IMAC step due to the geometry limitation of the column. Therefore, a proper scaled-up study based on same column height or contact time should be conducted in the future to improve the protein yield. Additionally, a new process also has been proposed to further improve the protein yield (Figure 6-1) where anion exchange Q sepharose HP is eliminated and sodium chloride is added both in the load and buffer solution in the Q XL polishing step (5).

SSX2 was also expressed in *P. pastoris* as a means to produce a delayed-type hypersensitivity (DTH) skin test reagent for monitoring SSX2-specific immune response from *E. coli*-produced SSX2 vaccine. *P. pastoris* has been reported to successfully secrete some recombinant proteins. However, secretion of some other recombinant proteins has proven to be difficult resulting in intracellular accumulation and low secretion yields (9, 14). Investigations in SSX2 secretion in *P. pastoris* indicated that SSX2 is also one of the recombinant proteins that are poorly secreted by *P. pastoris*. In the third part of this work, studies showed that SSX2 was misfolded and accumulated inside the endoplasmic reticulum (ER). Unfolded protein response (UPR) was induced and degradation of intracellular SSX2 was found possibly by the

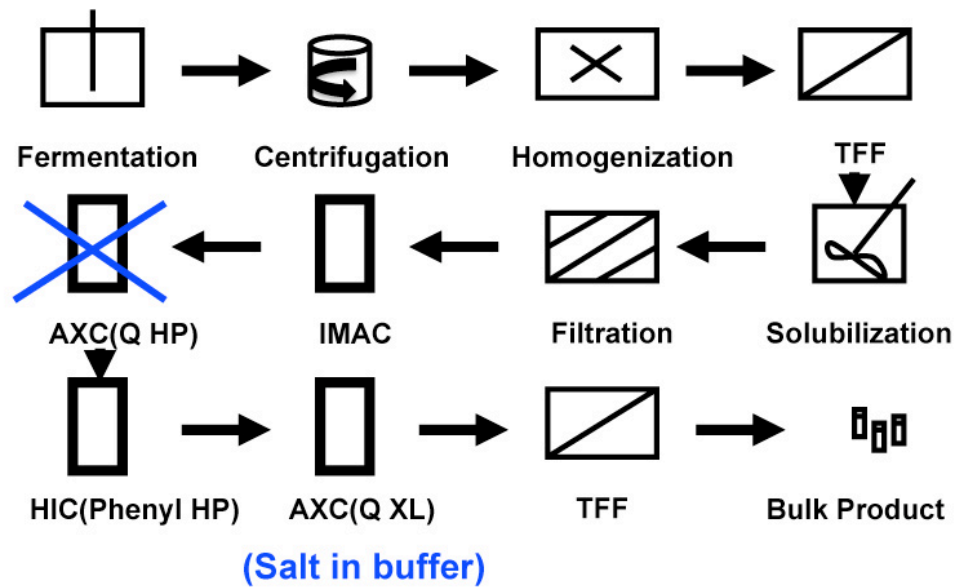


Figure 6-1. A suggested new SSX2 purification process for improving SSX2 production yield. The blue X and wording indicate the modifications made to the previous reported process.

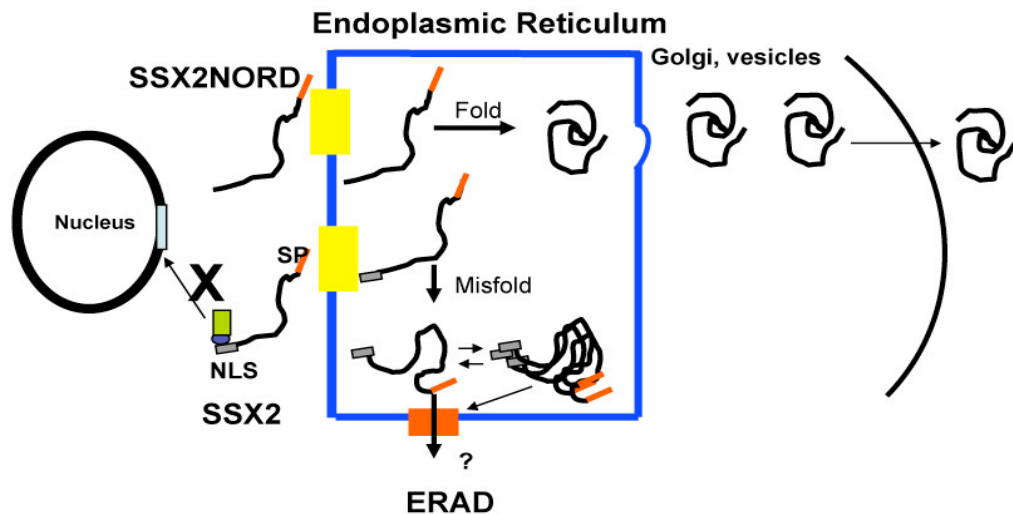


Figure 6-2. A simplified diagram showing the differences in secretion of SSX2 and SSX2 without nuclear localization signal (SSX2NORD) in *P. pastoris*.

activation of ER-associated degradation (ERAD) pathway to help the cells to cope with the ER stress (4). Deletion of C-terminal nuclear localization signal (NLS), however, significantly improves SSX2 secretion by improving its folding inside the ER (Figure 6-2). In addition, tagging NLS to a well-secreted protein, A33 single chain antibody, also decreased its secretion by 70%. Therefore, this study suggests that the NLS may inhibit or decrease recombinant protein secretion in *P. pastoris*. A fermentation protocol to produce SSX2 without NLS was later developed with yield of 21.6 mg/L that can be used as the DTH skin test reagent for SSX2 cancer vaccine. Even though, SSX2 secretion was improved by deletion of its NLS, its purification can be problematic due to its degradation in the culture media. To improve its stability, expression of SSX2 without NLS by *P. pastoris* SMD1168H, a vacuole peptidase A (*pep4*) defective strain (9), should be investigated in the future. Addition of protease inhibitors may also be considered as an alternative strategy to improve SSX2 stability in the culture media.

The last part of this work is the study of the proteome of culture media of *P. pastoris* at different fermentation conditions that attempts to improve recombinant protein purification and secretion in *P. pastoris*. Protein samples from culture media and two-dimensional (2D) gel spots at different fermentation conditions were subjected to ESI-MS/MS analysis and searched against a public fungi genome database downloaded in January 2009. A total of 34 proteins were identified directly from culture media and 28 gels spots were collected for further analysis. Most identified proteins have low isoelectric points ($pI < 6$) including naturally secreted proteins and some intracellular proteins. EXG1, Gas1p and some cell wall proteins comprised the majority of the secreted proteins being identified. Intracellular proteins such as alcohol oxidase and peroxiredoxin were also found in culture media from *P.*

pastoris induced with methanol.

The proteome data collected from this study can expand the biotechnology applications of *P. pastoris*. First, understanding host cell protein profile can reveal the identities of crucial contaminants with regard to the recombinant protein, providing strategies to remove these contaminants from the final product (15). Second, the use of the identified secreted proteins such as EXG1, Gas1P, Fet3, as fusion protein partners or their secretion signals can build a tool box to improve recombinant protein secretion. Third, deletion of the non-essential secreted genes can possibly enhance secretion of recombinant protein due to a lesser-saturated secretory pathway and also increase its predominance in the culture media. In June 2009, the whole genome sequence of *P. pastoris* has published (6). Future study should include searching all MS spectra against the new *Pichia* genome database to identify more proteins presented in the culture media. Studies of recombinant protein secretion using different secretion signals, such as EXG1, Gas1P and Fet3, should be conducted to provide new secretion signals and tools for recombinant protein secretion in *P. pastoris*.

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